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# ANNALS OF BOTANY

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# Physiological and Ecological Studies in the Analysis of Plant Environment

## III. The Interaction between Light Intensity and Mineral Nutrient Supply in Leaf Development and in the Net Assimilation Rate of the Bluebell (*Scilla non-scripta*)<sup>1</sup>

BY

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AND

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(Department of Botany, Imperial College, London)

With fourteen Figures in the Text

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## INTRODUCTION

THE first paper of this series (Blackman and Rutter, 1946) was concerned with an analysis by statistical methods of the importance of the light factor in governing the distribution of the bluebell (*Scilla non-scripta*) in deciduous woodland. In the sites examined it was demonstrated that where there was a closed canopy up to three-quarters of the variance of bluebell density could be accounted for in terms of the variation in shading. By fitting multiple regressions of density on the seasonal changes of light intensity it was also shown that the 'high' light phase during March and April, before leaf expansion in the overhead canopy, exerted a greater influence than the subsequent 'low' light phase of May and June at a time when the canopy was partially or fully expanded.

<sup>1</sup> Some of the results included in the paper were presented by the second author in a thesis for the Ph.D. degree of the University of London.

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The second paper (Blackman and Rutter, 1947) sought firstly to analyse the seasonal growth and development of the bluebell under woodland conditions, and secondly to determine the effects of shading and mineral nutrient supply on bulb, flower, and leaf growth of plants grown experimentally under a range of screens. Both series of experiments revealed that the rate of seasonal growth was extremely slow. Even in full daylight the annual gain in plant weight ranged only from a twofold to a 3.7-fold increase, immature non-flowering plants having a greater growth rate than mature flowering plants.

The marked sensitivity of the bluebell to shading, indicated by the initial woodland observations, was confirmed in the field experiments. A reduction of the light intensity to less than half daylight may decrease the growth-rate, while at a value of 0.11 daylight little increase in plant size takes place.

Even under conditions of a low level of mineral nutrient supply, additional nitrogen, phosphorus, and potassium has little effect on the growth of the bluebell. When small increases were recorded these were related to the light intensity, since gains in full daylight were statistically significant while at a level of 0.22 daylight they were not.

This, the third paper in the series, relates to a further analysis of the effects of light intensity and mineral nutrition on leaf development and function, and particularly the influence of these factors on net assimilation rate.

## EXPERIMENTAL PROCEDURE

### *Design of experiments*

The design and general planning of the field experiments has been given in some detail in a previous paper (Blackman and Rutter, 1947). To recapitulate briefly: graded, dormant bulbs were planted in the autumn on an infertile gravel soil; subsequently in the spring as soon as the leaves emerged above ground, the appropriate plots were shaded with screens covered with either white butter muslin, black lawn, or sheets of perforated zinc. In each experiment there were three to four levels of light intensity, ranging from full to 0.11 daylight. Superimposed on the light treatments there were in the largest experiments eight combinations of mineral nutrient supply, viz. C, N, P, K, NP, NK, PK, and NPK, making in all twenty-four treatments.

When the screens were placed on the plots an initial random sample was taken from each plot, and after washing the plants to remove soil particles they were divided into bulb plus roots, leaves, and inflorescence. These divided samples were then dried at 100° C. and weighed separately. During the course of the experiment similar samples were obtained at intervals and treated in the same way.

### *Estimation of leaf-area*

Because of the need to carry out the determinations of leaf-area before the leaves wilted, it was impossible with the labour available to measure the area

of all the leaves from all the samples. Since preliminary determinations showed that light intensity pre-eminently controlled the size and shape of the leaves, area determinations within each light level were made on sub-samples from half the plots. During the 1938 and 1939 investigations the length and the maximum width were determined for each leaf in the sub-sample. Simultaneously, from each of the sub-samples the outline of some dozen measured leaves was traced on paper by placing the leaves between two sheets of glass and illuminating from below. The area of each traced leaf was then estimated by means of a planimeter and the correction factor for converting length  $\times$  maximum width into an estimate of leaf-area determined. Subsequently the whole of the leaves in the sub-sample were bulked, dried, and weighed. With these data it was possible to arrive at the ratio of leaf-area per gramme of dry leaves for the sub-sample, and to apply this to the total dry leaf weights.

In 1940 a quicker and simpler method of estimating leaf-area was employed. By means of a cutter consisting of two parallel safety razor blades, which could be set at any desired distance apart, the tip and base of each leaf in the plot sub-sample were removed. The area of the remaining portions, consisting of trapeze-shaped pieces of equal length, could be calculated as the product of length and mean width. The sub-sample of trapeze-shaped strips was then dried and as before the ratio of leaf-area to unit dry weight determined. By applying these factors to the corresponding total leaf-weight data, the total leaf-areas were estimated.

As far as the precision of these estimates is concerned, the standard errors of the area per gramme factor on an individual plot basis were 4.13 per cent. and 1.93 per cent. of the mean in 1938 and 1939 respectively and 2.08 per cent. in 1940.

#### EXPERIMENTAL RESULTS

##### *The effects of varying light intensity on leaf-area*

In the second paper (Blackman and Rutter, 1947) it was demonstrated that during the active growth phase, variations in the light level did not significantly affect leaf weight in any of the six experiments. Subsequent to flowering, shading retarded the onset of leaf senescence, and in consequence at the lower light intensities the leaf weight was significantly greater.

In spite, however, of the independence of light intensity and total leaf-weight during the period of active growth, Figs. 1 and 2 clearly show that light intensity has a marked effect on leaf-area per plant. In both the 1938 and 1939 experiments with mature flowering bulbs a reduction in the light intensity increased the leaf-area. Examination showed that the shade leaves were thinner and the cells larger, and this is reflected in the ratio of unit leaf-area to unit leaf weight. The results for experiment III 1938 and experiment IV 1939 are given in Table I. It is seen that in 1938 the influence of varying light intensity on the area weight ratio becomes progressively greater with time: in 1939 this is less evident.

Since light intensity has little effect on leaf-weight, it follows that any changes in leaf area will be directly proportional to variations in the ratio of

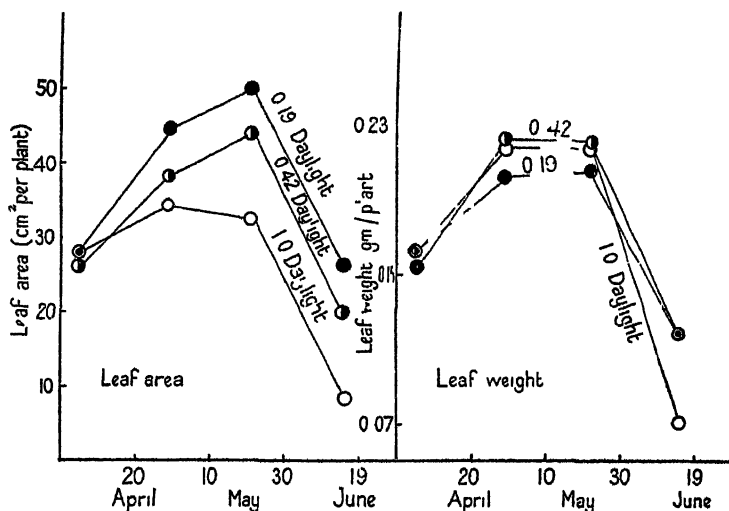


FIG. 1. The effects of varying light intensity on the seasonal changes in leaf-area and leaf-weight of the bluebell (expt. III, 1938).

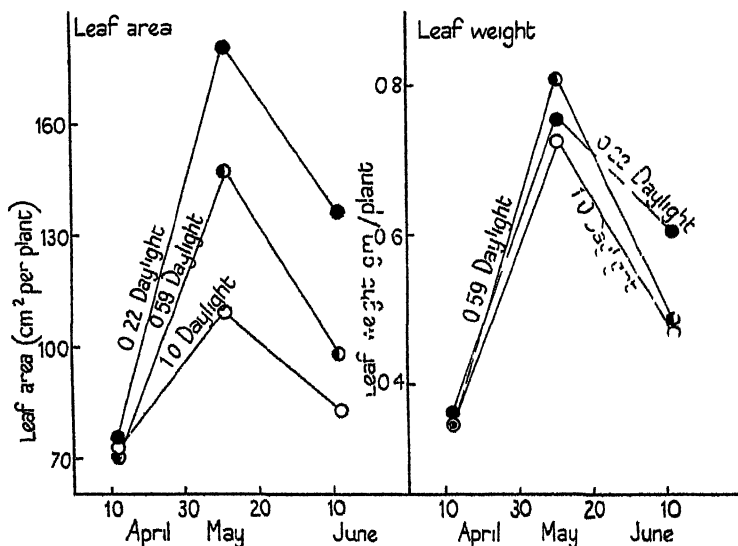


FIG. 2. The effects of varying light intensity on the seasonal changes in leaf-area and leaf-weight of the bluebell (expt. IV, 1939).

leaf-area to leaf-weight. This ratio can therefore serve as a comparative measure between experiments. The results from three experiments where there were four levels of light intensity are seen in Fig. 3. The ratios of area

to leaf-weight show a progressive rise with falling light intensity. In fact over the range of full to 0.11 daylight the ratio is linearly proportional to the logarithm of decreasing-light intensity.

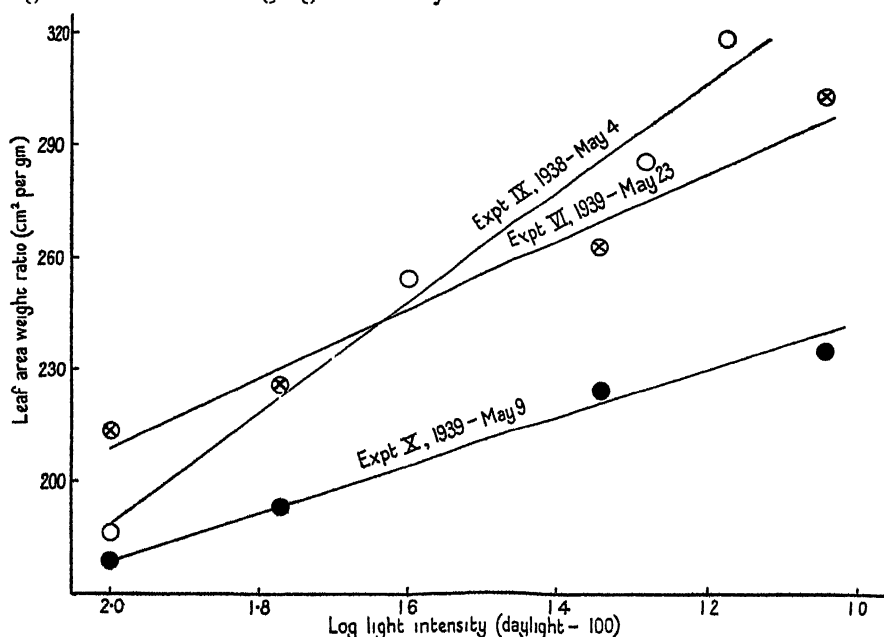


FIG. 3. The effects of varying light intensity on the ratio of unit leaf-area to unit leaf-weight in the bluebell.

TABLE I

*The Effects of Varying Light Intensity on the Ratio of Leaf-Area to Leaf Dry Weight*

*Ratio of Leaf-Area to Leaf-Weight (sq. cm. per gm.)*

*Experiment III, 1938*

*Date of sampling.*

April 29.		May 22.		June 15.	
Light intensity.	Ratio.	Light intensity.	Ratio.	Light intensity.	Ratio.
1.00*	161	1.00	157	1.00	131
0.39	175	0.42	200	0.45	169
0.18	218	0.19	240	0.20	227

Significant difference between treatments = 17 ( $P = 0.05$ )

*Experiment IV, 1939*

*Date of sampling.*

Light intensity.	May 11.	June 12.
1.00	151	178
0.59	182	204
0.22	239	227

Significant difference between treatments = 15 ( $P = 0.05$ )

\* Full daylight = 1.00.

*The effects of varying light intensity on net assimilation rate*

Since in a number of experiments changes in total plant weight and leaf-area were simultaneously measured, the 'net assimilation rate', as defined by Gregory (1926), or the 'unit leaf rate' on an exponential basis of Briggs, Kidd, and West (1920), can be calculated. The formula used was:

$$\frac{(W_2 - W_1)(\log_e A_2 - \log_e A_1)}{(A_2 - A_1)(t_2 - t_1)},$$

where  $W_2$  and  $W_1$  represent the final and initial weight per plant at the times of the two sampling occasions ( $t_2$  and  $t_1$ ), and  $A_2$  and  $A_1$  the leaf-areas. The results have been expressed as gm. of plant weight per sq. dm. of leaf per week. It should, however, be noted that this equation is only an approximation since it assumes that changes in weight are linearly related to changes in leaf-area.

In experiment III, 1938, results for mature flowering bulbs are available for three consecutive periods covering the seasonal growth. In this, the first of the field experiments, the screens were covered with black 'lawn' and black gauze which faded slowly under the action of light and rain. As, however, the transmission factor of the screens was estimated frequently by means of photo-electric cells (for details see Blackman and Rutter, 1947), the average shading over each period can be calculated.

TABLE II

*The Effects of Varying Light Intensity on Net Assimilation Rate (gm. per sq. dm. per week) at Successive Periods in the Season*

April 4-29.		Experiment III, 1938		May 22-June 15.	
Light intensity.*	N.A.R.	Light intensity.	N.A.R.	Light intensity.	N.A.R.
1.00	0.625	1.00	0.627	1.00	0.539
0.40	0.382	0.45	0.378	0.53	0.643
0.18	0.211	0.20	0.140	0.21	0.030
Significant difference ( $P = 0.05$ )	0.163	--	0.165	-	n.s.

\* Full daylight 1.00.

The results set out in Table II show that over the first two periods reductions in the light intensity have significantly decreased the net assimilation rate. In the third period the results are not significant, but this is not surprising since by the end of the period senescence of the leaves had set in, with a consequent withering of the leaf tips and irregular yellowing of the remainder. As a result the estimates of the assimilating area were liable to error, and this variability is reflected in the high standard error of the mean net assimilation rate—namely, 334 per cent. In fact it is not legitimate to apply the analysis of variance to such material (Cochran, 1938).

In Fig. 4 the net assimilation rate has been plotted against the logarithm of the light intensity for the first two occasions. It is seen that in both instances

there is a remarkably close fit to a straight line-regression. Moreover, from the fitted regressions it is possible by extrapolation to determine the level of light intensity where leaf assimilation is balanced by respiration losses of the

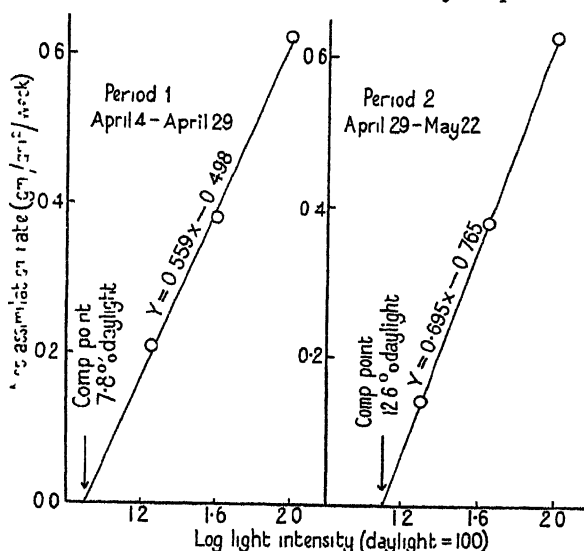


FIG. 4. The effects of varying light intensity on the net assimilation rate of the bluebell (expt. III, 1938).

whole plant. The values of the 'compensation point' obtained are 0.078 daylight for the April period and 0.126 for the May period.

TABLE III

*The Effects of Varying Light Intensity on Net Assimilation Rate (gm. per sq. dm. per week) at Successive Periods in the Season*

*Experiment IV, 1939*

Light intensity (Daylight 1.00).	April 12 May 11.	May 11-June 12.
1.0	0.488	0.591
0.59	0.421	0.452
0.22	0.074	0.192
Significant difference ( <i>P</i> 0.05)	0.149	0.168

A similar experiment was carried out in 1939 on mature flowering bulbs, but in this instance observations were completed before the onset of leaf senescence. The range of light intensities employed was somewhat different since butter muslin and perforated zinc sheeting, which gave a constant degree of shading, replaced the black lawn and gauze. With these levels, in neither the first nor the second sampling period is the difference in net assimilation rate between daylight and 0.59 daylight significant. Nevertheless, reference to Fig. 5 shows that there is again a close relationship between the

net assimilation rate and the logarithm of the light intensity. As before, estimates of the compensation points can be obtained by extrapolation of the regression equations. For the first period the value is 0.161 of daylight and for the later period the value is somewhat lower viz. 0.107 daylight.

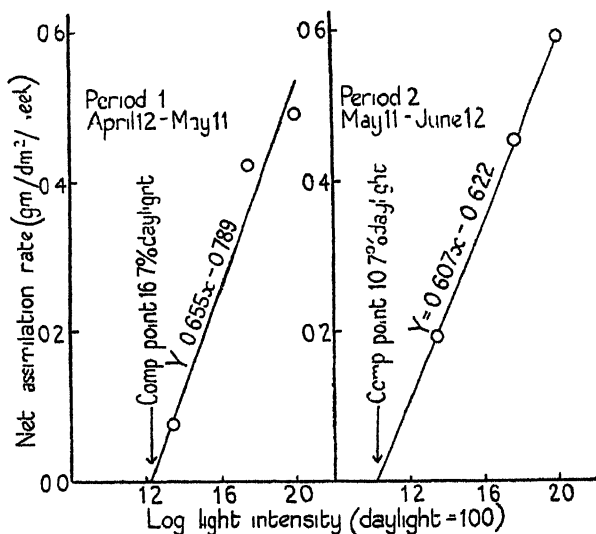


FIG. 5. The effects of varying light intensity on the net assimilation rate of the bluebell (expt. IV, 1939).

In the third experiment (expt. V, 1940), carried out with mature bulbs, data are only available for a single period. Once more the linear proportion between net assimilation rate and the logarithm of the light intensity is seen in Fig. 6, and the value obtained for the compensation point (0.081 daylight) is lower than in the previous experiments. Table IV demonstrates that the reduction in light intensity from 1.00 to 0.68 of daylight has caused a significant decrease in assimilation.

In addition to results on flowering plants, comparable data are available from one experiment in 1939 on small plants which had not yet reached the flowering stage. Fig. 6 once more reveals the now expected linear relationship between net assimilation rate and falling light intensity. The compensation point of 0.095 is also in line with the previous estimates. Table IV shows that each reduction in light intensity has significantly reduced the assimilation rate.

#### *The effects of varying the periods of shading on growth assimilation, and relative leaf-area*

In the field experiments so far described in this series of papers the bluebell plants were subject to constant levels of reduced light intensity over the whole season. On the other hand, in deciduous woodland, between early April and June the degree of shading is not constant; there is a very marked increase in shading from the high light phase when the trees are dormant in early

spring to the low light phase when the overhead canopy has fully expanded (Blackman and Rutter, 1946).

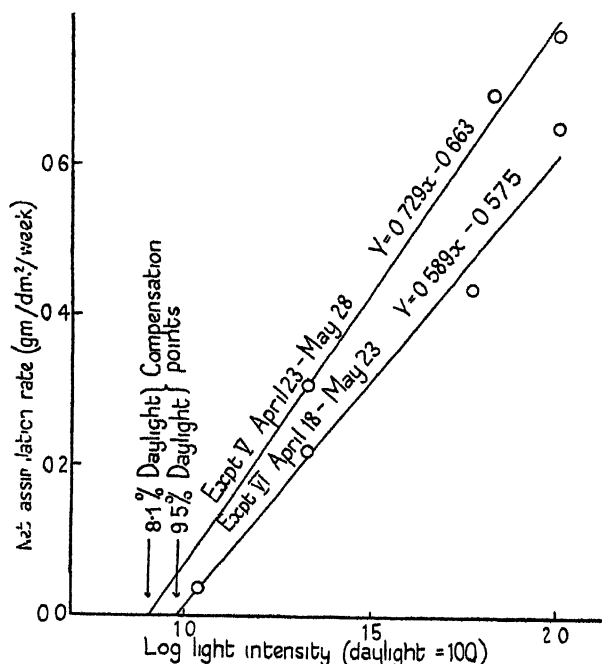


FIG. 6. The effects of varying light intensity on the net assimilation rate of the bluebell (expt. V, 1940, expt. VI, 1939).

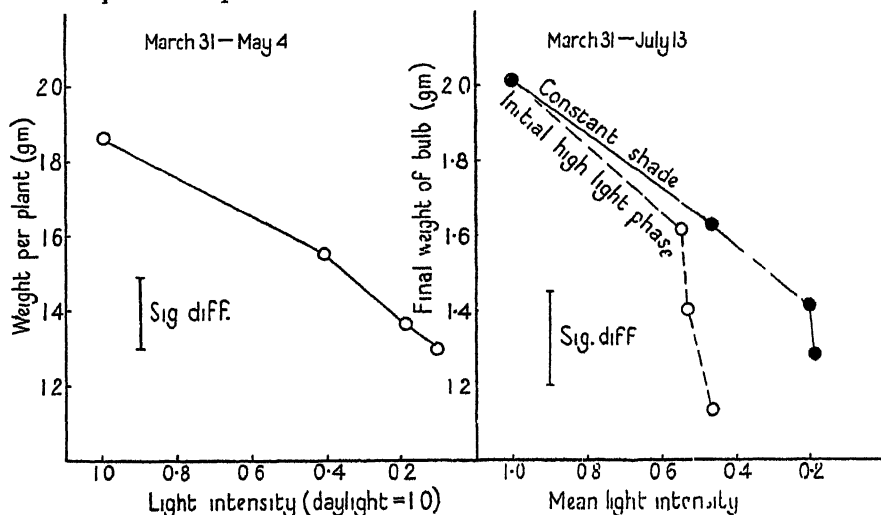
TABLE IV

*The Effects of Varying Light Intensity on Net Assimilation Rate*  
(gm. per sq. dm. per week)

Experiment V, 1940 April 23 May 28.		Experiment VI, 1939 April 18-May 23.	
Light intensity (daylight 1.0).		Light intensity (daylight 1.0).	
1.00	0.776	1.00	0.629
0.68	0.699	0.59	0.435
0.22	0.307	0.22	0.219
--	--	0.11	0.038
Significant difference (P 0.05)	0.056	—	0.085

In order therefore to simulate experimentally this seasonal increase in shading under woodland conditions, plot experiments were carried out in 1938 and 1939. The essential design in both years consisted of shading with three levels of reduced light intensity (a) one series of plots as soon as the larch trees (*Larix europaea*) in the neighbourhood 'broke' in early April, and (b) delaying shading the second series until the oaks (*Quercus robur*) started to expand their leaves a month or so later. In this way it is possible to compare the growth of plants receiving (a) constant shading during the season and

(b) a 'high' light phase followed by a 'low' light phase. Moreover, since Daxer (1934) claims that the assimilation rate and compensation point are conditioned by the previous level of light intensity to which the plants have been subjected, the leaf areas were determined as well as the changes in weight of bulb, leaf, and inflorescence. Thus it is possible to calculate the net assimilation rates, and by relating them to the varying light intensity to assess the possible after-effects of a 'high' and 'low' light phase on both the assimilation rate and the compensation point.



FIGS. 7A and 7B. Fig. 7A. The effects of varying light intensity on the growth of the bluebell in the early spring. Fig. 7B. The effects of varying light intensity on final bulb size where the mean light intensity was either made up of a constant degree of shading during the season or is composed of an initial high light phase (March 31–May 4), when the plants received full daylight, followed by a subsequent period of low light intensity (expt. IX, 1938).

In the first of the experiments (exp. IX, 1938) the screens were placed on the 'larch' series (constant shading) on March 31, and on the 'oak' series (variable shading) on May 4. Random samples of plants were taken from the plots on March 31, May 4, May 30, and again when the leaves had died back on July 31. The screens consisted of single or double layers of black lawn or gauze, and as stated earlier these tended to fade in the course of the experiment. In consequence the light intensities quoted are the mean levels between the beginning and end of each experimental period.

There was, however, no seasonal fading in experiment X (1939) since the screening materials were changed to butter muslin and perforated zinc. The plots were sampled on three occasions, namely, April 11, when the first series of screens were placed in position, May 9 when the second series of plots were shaded, and finally on August 2, by which time there remained only dormant bulbs.

The effects of reduced light intensity on plant weight during 1938, for the initial period (March 31–May 4) and over the whole season (March 31–July 13), are shown in Figs. 7A and 7B. It is seen that both during April and over the

whole season shading has markedly reduced plant size. It is also to be observed that those plants which were not shaded during April appear to be more susceptible to reduced light intensity, i.e. when the mean degree of shading is made up of a high and low light phase, the shading effect is greater.

Since the levels of light intensity are not the same in both series it is necessary, in order to test the significance of this differential effect, to fit regressions of plant weight on light intensity for the two series. Since the highest light intensity of full daylight over the whole season is common to both series, regressions of final plant weight ( $y$ ) on the logarithm of mean light intensity ( $x$ ) would be expected to intersect where  $x = 2.0$  if full daylight is expressed as 100. Allowing for this limitation it can be shown that the terms of the two regressions, one for the constant shade series and one for the high and low light phase, of the form

$$Y = b(x-2) + a$$

can be obtained by solving the following equations:

$$\begin{aligned} \bar{y}_1 + \bar{y}_2 - 2a + b_1(2 - \bar{x}_1) + b_2(2 - \bar{x}_2) &= 0, \\ \sum_n \frac{x_1 y_1}{n} - 2\bar{y}_1 + a(2 - \bar{x}_1) + b_1 \left( 4\bar{x}_1 - \frac{\sum x_1^2}{n} - 4 \right) &= 0, \\ \sum_n \frac{x_2 y_2}{n} - 2\bar{y}_2 + a(2 - \bar{x}_2) + b_2 \left( 4\bar{x}_2 - \frac{\sum x_2^2}{n} - 4 \right) &= 0, \end{aligned}$$

where the suffixes 1 and 2 denote the constant shading series and the high and low phase series respectively, and  $a$  is the common value of  $y_1$  and  $y_2$  when  $x = 2$ .

The errors about the two separate regression lines and about a common line can be calculated for each block separately, and an analysis of these data (see Table V) shows that there is a significant advantage to be gained by fitting six regressions rather than three. Thus the two series differ significantly in the relation between plant weight and light intensity. Combining the corresponding regressions from each block the relationships given below are obtained; it is clear that for a given average level of light intensity during the season the constantly shaded plants make better growth than those receiving full daylight initially.

Constant shading series  $Y_1 = 0.968x_1 + 0.084$ .

High and low light phase series  $Y_2 = 2.221x_2 - 2.42$ .

TABLE V

*Errors about Regressions of Final Weight on the Logarithm of Mean Seasonal Light Intensity*

	Degrees of freedom.	Sum of squares.	Variance.
6 regressions (two series of shading)	15	0.3851	0.0257
3 " (common line for each block)	18	1.1947	—
Advantage of fitting 6 regressions	3	0.8096	0.2699
$F = 10.50^{**}$			

A further analysis of this light effect can be obtained from a consideration of the data for the period May 4–July 31, i.e. the period when screens were placed on the plants which had hitherto been grown in daylight. Since the plants of the constant shading series had been subjected to four levels of light intensity up till May 4, the mean plant weight at this time differed significantly (see Fig. 7A). Therefore the only comparable quantity that can be used as a measure of growth for all treatments during the second period is  $W_2/W_1$ , where  $W_2$  is the mean plant weight on May 30 and  $W_1$  the weight on May 4.

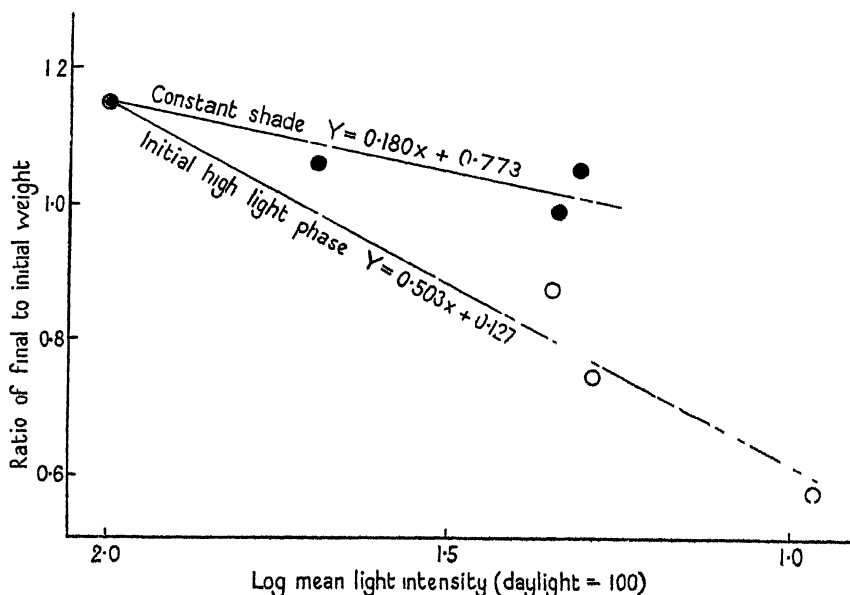


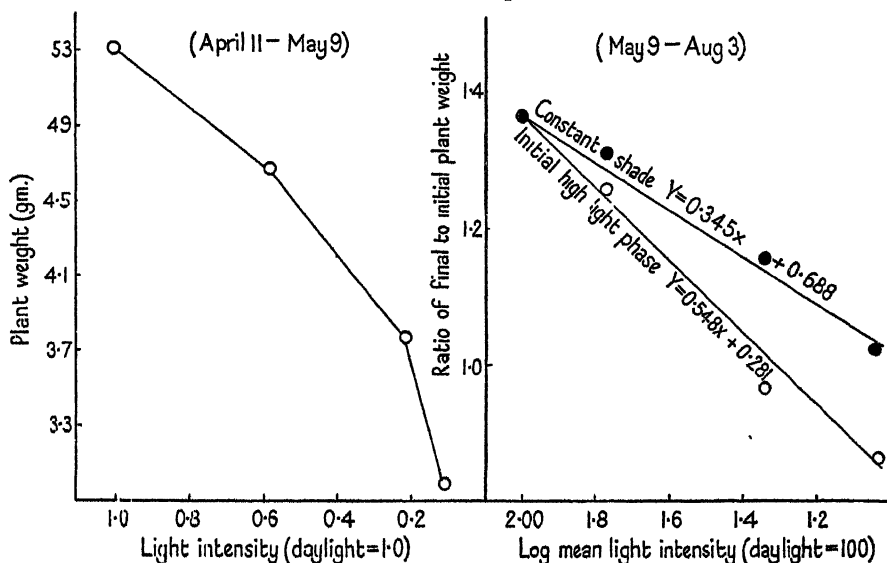
FIG. 8. The effects of varying light intensity on the relative change in weight of bluebell plants which prior to the experimental period have either been grown in full daylight or been subjected to varying degrees of shade (expt. IX, 1938).

The method of regressions as already described was used to compare the constant shading series with the high and low light series, and an analysis of variance showed that, as before, the regressions of the logarithm of light intensity against change in weight differed significantly for the two series. These regressions are set out in Fig. 8, and it is clear that, for a given reduction in the light intensity, plants shaded during April made more growth in May than those previously exposed to full daylight in April.

The effects of varying light intensity on plant weight during the initial shading period of the constant shade series of the 1939 experiment are given in Fig. 9A. There is once more a marked decrease in growth with falling light intensity. The subsequent influence of the initial low light, as against the high light phase, on the relative growth of shaded plants during the rest of the season is shown in Fig. 9B, where regressions of the ratio  $W_2/W_1$  against light intensity have been fitted to the data. Inspection indicates that the results are

similar to those of 1938; plants previously shaded are subsequently less sensitive to reduced light intensity than plants receiving full daylight in the first period. Statistical analysis of the data reveals, however, that the error is high and the regressions do not differ significantly.

From both the 1938 and 1939 experiments it is possible to obtain further evidence of the relationship between net assimilation rate and light intensity. The net assimilation rates, corresponding to the changes in weight in the initial period of shading (vide Figs. 7A and 9A), are shown in Fig. 10. Further confirmation is obtained of the linear relationship between the assimilation rate and the logarithm of the light intensity. It is significant that the estimates of the compensation point obtained by extrapolation of the fitted regressions give identical values for the compensation point.



FIGS. 9A and 9B. Fig. 9A. The effects of varying light intensity on the growth of the bluebell in the early spring. Fig. 9B. The effects of varying light intensity on the relative growth rate of plants which prior to the experimental period have either been grown in full daylight or been subjected to varying degrees of shade (expt. X, 1939).

The 1938 data for the second period (May 4-30) also allow of a comparison between the net assimilation rates of plants previously shaded or not shaded in the initial April period. The data, given in Table VI, indicate that where the light values are comparable the net assimilation rates are not differentially affected by the previous light history. Statistical analysis of fitted regression lines confirms this assumption; the common regression,  $Y = 0.546x - 0.497$ , on extrapolation gives an estimate of 0.081 daylight for the compensation point.

As the influence of the preliminary light treatment on subsequent growth cannot be accounted for in terms of changes in net assimilation rate, some other explanation must be advanced. Rate of growth is made up of the product of net assimilation rate and total leaf-area. It follows therefore that

the changes in growth-rate observed may well be due to the variations in total leaf-area brought about by the light treatments, i.e. the influence of preliminary shading on the ratio of total leaf-area to total plant weight or 'relative leaf-area'.

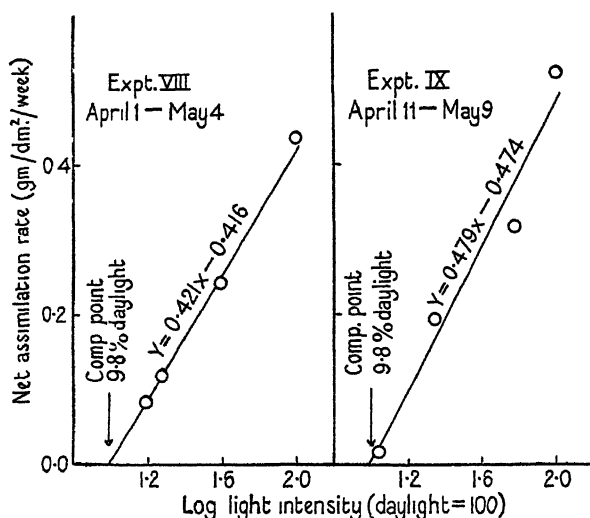


FIG. 10. The effects of varying light intensity on the net assimilation rate of the bluebell.

From the data of experiment IX, such relative leaf-areas can be calculated for the end of the first period when the plants had been subjected to light intensities ranging from full daylight to 0.15 daylight, and again at the end of the

TABLE VI  
*The Effects of Varying Light Intensity on Net Assimilation Rate*  
(gm. per sq. dm. per week)  
*Experiment IX, 1938*

	Light intensity (daylight = 1.0).	May 4-30.
Plants previously shaded (April 1–May 4)	1.00	0.652
	0.45	0.326
	0.18	0.166
	0.18	0.153
Plants not previously shaded	1.00	0.652
	0.21	0.154
	0.17	0.152
	0.06	0.042
Significant difference ( $P = 0.05$ )	—	0.259

second period when all plants had been shaded. These results are given in Fig. 11. It is seen that the plants initially exposed to a reduced light intensity have a higher relative leaf-area than the plants receiving full daylight. This extra leafiness holds throughout the second period, though at the end it is not so great as at the beginning, since shading the hitherto unshaded plants has led to a higher leaf-area per unit leaf-weight.

Thus it is clear that the main effect of preliminary shading is to increase the leaf-area of the shaded plants such that during the second period their growth rate under reduced light intensity exceeds that of the plants initially receiving full daylight and possessing, in consequence, a smaller relative leaf-area. There is therefore no need for the postulate of Daxer (1934) that

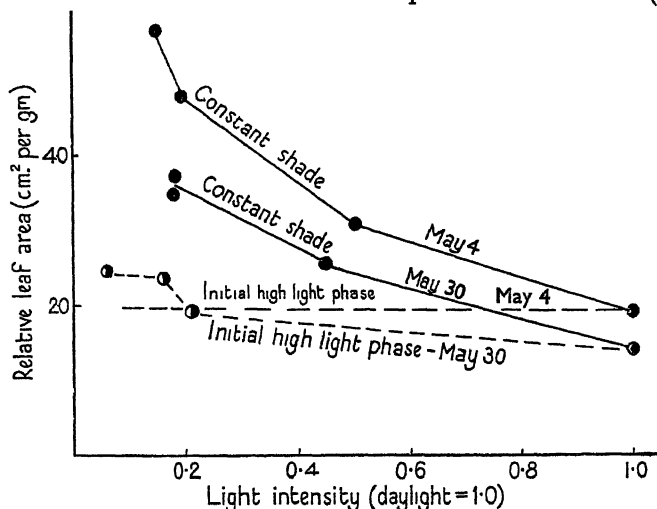


FIG. 11. The effects of varying light intensity on the changes in relative leaf-area (total leaf-area over total plant weight) of bluebell plants which prior to the experimental period (May 4-30) have either been grown in full daylight or been subjected to varying degrees of shade (expt. IX, 1938).

adjustment to preliminary shading is invariably a question of internal factors affecting the assimilatory-respiratory complex. In experiment X (1939) there is the same trend. Shaded plants at the end of the first period have a higher relative leaf-area than plants receiving full daylight.

*The effects of mineral nutrient supply on net assimilation rate, relative leaf-area and plant weight*

In only one of the experiments (exp. III, 1938), and then only over one period, is there any significant effect of nutrient supply on net assimilation rate expressed in terms of leaf-weight (see Table VII). In no experiment is there any interaction between light intensity and nutrient level.

In the second paper of this series the largest effects of mineral nutrient supply on total plant weight were recorded for experiment IV, and since the present evidence does not indicate that such changes can be accounted for in terms of variations in net assimilation rate, the effects of nutrient supply on the relative leaf-area need examination. The data for changes in plant weight, net assimilation rate, and relative leaf-area for experiments III-V are therefore set out in Tables VII-IX.

From Table VII it would seem that the increased growth due to additional nitrogen can be attributed to the increase in net assimilation rate. On the

TABLE VII

*The Effects of Varying Mineral Nutrient Supply on Plant Weight, Net Assimilation Rate, and Relative Leaf-area*

*Experiment III, 1938*

	Dry wt. of plant (gm.).	N.A.R. (gm./gm./week).		Relative leaf-area (cm. <sup>2</sup> /gm. plant weight).
		April 4-29.	April 29- May 22.	
Nitrogen	1.76*	0.62	0.87*	24.2
No Nitrogen	1.69	0.74	0.54	23.6
Phosphorus	1.72	0.72	0.64	25.2*
No phosphorus	1.73	0.64	0.77	24.6
Potassium	1.74	0.72	0.68	24.2
No potassium	1.71	0.64	0.73	23.7

\* Pairs of figures differ significantly.

other hand, additional phosphorus appears to have decreased the ratio of leaf area to plant weight without this being reflected in any change in plant weight.

TABLE VIII

*The Effects of Varying Mineral Nutrient Supply on Plant Weight, Net Assimilation Rate, and Relative Leaf-area*

*Experiment IV, 1939*

	Dry wt. per plant (gm.).	N.A.R. (gm./gm./week).		Relative leaf-area (cm. <sup>2</sup> /gm. plant weight).	
		April 12- May 11.	May 11- June 12.	April 12.	May 11- June 12.
Nitrogen	5.34*	0.69	0.67	29.6	31.7*
No nitrogen	4.95	0.61	0.78	29.1	27.4
Phosphorus	5.36*	0.71	0.59	30.1	30.0
No phosphorus	4.93	0.70	0.75	28.6	29.2
Potassium	5.33	0.69	0.61	31.5	29.8
No potassium	4.96	0.69	0.77	27.2	29.4

\* Pairs of figures differ significantly.

From the results of experiment IV given in Table VIII it can be concluded that the increase in plant weight due to additional nitrogen is related to the increase in the relative leaf-area. In contrast, the effect of phosphorus in increasing plant weight is not reflected in any significant increase either in assimilation rate or relative leaf-area. Again in experiment V (Table IX), although nitrogen has increased the relative leaf-area, it has not in turn increased total plant weight. There is, however, agreement for the phosphorus response both in plant weight and the relative leaf-area.

It is not altogether surprising that there is some lack of correspondence between the changes in weight, assimilation rate, and relative leaf-area. All the mineral nutrient effects are small and since the experimental errors are not in all instances low, changes in one quantity may not be reflected in significant changes of the others.

TABLE IX

*The Effects of Varying Mineral Nutrient Supply on Plant Weight, Net Assimilation Rate, and Relative Leaf-area*

*Experiment V, 1940*

	Dry wt. per plant (gm.).	N.A.R. (gm./gm./week).	Relative Leaf-area (cm. <sup>2</sup> /gm. plant weight).	
		April 23- May 28.	April 23.	May 28.
Nitrogen	2.02	1.46	36.4*	34.4*
No nitrogen	1.90	1.51	34.3	33.1
Phosphorus	2.07*	1.49	35.8	34.3*
No phosphorus	1.86	1.48	34.9	33.2
Potassium	1.92	1.50	35.4	34.2
No potassium	2.01	1.47	35.3	33.3

\* Pairs of figures differ significantly.

### DISCUSSION

This further analysis of the environmental factors which control the development and growth of the bluebell both confirm and amplify the findings of the previously reported field and woodland studies (Blackman and Rutter, 1946 and 1947). All the results stress the sensitivity of the bluebell to relatively slight shading. The present investigation shows with a new precision how the light factor operates in controlling the growth of higher plants, since no previous investigations concerned with the effects of shading have included both measurements of net assimilation rate and determinations of the relative leaf-area.

The most striking feature of the results is the demonstration for the first time of an exact linear relationship between net assimilation rate and the logarithm of the light intensity over a range of full daylight to 0.11 daylight. From Figs. 4, 5, 6, and 10 it is clear that the agreement is very close for the nine sets of observations. In Fig. 12 the fitted regressions relating the logarithm of light intensity to net assimilation rate have been redrawn for the *untransformed* values of light intensity. Since in full daylight the curves are still rising, it can be inferred that the net assimilation rate of the bluebell would be increased still further at higher light intensities than those ruling in the spring.

On the other hand, such increased light intensities would necessarily involve higher temperatures, and these might operate adversely if they exceeded the optimum for maximum assimilation rate. For the bluebell this maximum is unknown, but values have been recorded for other spring-flowering or woodland plants. According to Mudrack (1935) the optimum for *Ranunculus Ficaria* is attained at 8-10° C., while for *Anemone nemorosa* maximum assimilation takes place over a range of 10-20° C. with a 40 per cent. reduction when the temperature is raised to 30° C. (Lundegardh, 1931).

Since *A. nemorosa* and more especially *R. Ficaria* produce leaves and flowers earlier in the spring than the bluebell (Salisbury, 1916), it is probable that the

optimum temperature limits of the bluebell at least equal those for *A. nemorosa*—if they are not higher. If this supposition is correct, then as temperatures in the spring rarely approach 20° C. in April and the first half of May a higher intensity with the attendant higher temperature could still operate in raising the assimilation rate.

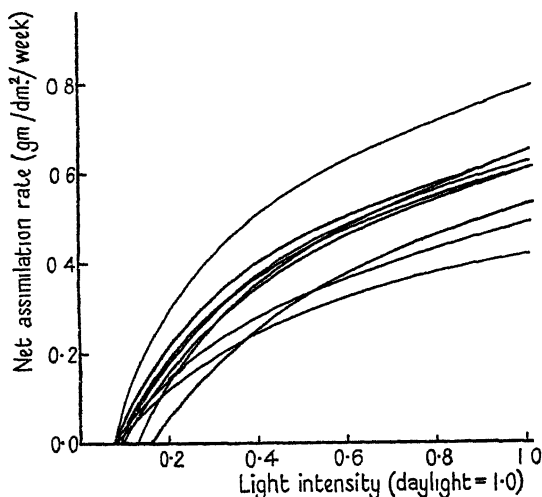


FIG. 12. The effects of varying light intensity on the net assimilation rate of the bluebell: individual curves for each experimental period.

Although in all experiments this linear relationship between the logarithm of the light intensity and net assimilation rate holds for the active growth phase, the rates of assimilation vary considerably between experiments. In full daylight the maximum and minimum values range from 0.776 to 0.433 (expts. V and IX) with a mean value of 0.594.

This mean value is only a little above the average figure of 0.552, cited by Heath and Gregory (1938) and based on net-assimilation-rate estimates made by different workers for various species, including tropical plants. In discussing the factors responsible for the small variations in assimilation rate during the period of most active growth, Heath and Gregory were of the opinion that it was not apparent that leaf structure is necessarily a determining factor. They put forward the view that the total amount of light received per day might well account for the similarity of the results, since with changes in latitude the mean light intensity might be offset by the length of day. On the other hand, these authors considered that this invariable light limitation was not supported by the investigations of Bolas et alia (1938) on the growth of tomatoes in glasshouses, since these workers found that the average net assimilation rate during winter days was of the same order as that ruling in the summer months.

In later experiments on the tomato, conducted by Goodall (1945), it was found that tomatoes under glass exhibited wide variations in net assimilation

rate (on a leaf-weight basis) between summer and winter. At first sight these two sets of results seem in direct conflict, but the discrepancies are in part due to the different experimental procedure adopted. Bolas et alia were concerned only with the net assimilation of the shoot, and their experimental period was kept constant at 7 hours of daylight. Goodall, on the other hand, sampled whole plants, and in this connexion Williams (1936, 1945) has demonstrated that for oats the net assimilation rate (on a leaf-weight basis) is very different for whole plants as against shoots alone. Moreover, Goodall's experimental period was 24 hours and therefore not only included the day and night effects but also differences in day length between summer and winter.

As Gregory (1926) has pointed out, the relationship of net assimilation rate to temperature and the light factors is complicated by the interdependence of these variables. Goodall's results confirmed that changes in assimilation rate during the year were positively linked with average daily light intensity, the mean day length, the day and night temperatures, and negatively related to the saturation deficit of the atmosphere. Nevertheless in spite of the interdependence of these factors, Goodall demonstrated that mean light intensity was the only factor to give a significant effect when the other variables had been eliminated. Moreover, it should be pointed out that the seasonal differences of light intensity within the greenhouse did not directly reflect the changes in the open, for during the summer months the glass was sprayed with 'summer cloud' to reduce the transmission of both light and heat rays; thus the full effect of increased light intensity during the summer could not operate.

Watson (1947), examining comprehensively the seasonal drift in the net assimilation rate for a number of crops in the field, was unable to demonstrate a significant light effect where the varying intensity and duration of daylight were integrated by means of a Callender recorder, an instrument which does *not*, however, differentiate between infra-red and visible radiation.

In contrast, the relationship between temperature and assimilation rate was more definite. As Gregory (1926) had found for barley, there was evidence in potatoes of a negative correlation with minimum night temperature. But for sugar-beet the correlation was positive, while in the case of wheat mean daily temperature and assimilation were significantly linked. Watson concludes tentatively that these surprising differences between the three plants may be due to the indirect effects of temperature, since high temperatures will be associated with sunny dry periods—periods when the available moisture may be minimal and cause differential wilting between species.

Apart from the temperature and light factors and their effects on the assimilation rate, there remains the question of the part played by internal factors within the plant. Ballard and Petrie (1936) and Williams (1937, 1939, and 1946) claim that the net assimilation rate, expressed in terms of leaf weight or leaf protein, is dependent upon the stage of development of the plant. This drift with time in the assimilation rate is least evident during the period of leaf development, but once the maximum leaf area has been obtained

the rate tends to fall. Goodall (1946) in his further studies of the tomato concludes that in newly formed leaves the net assimilation rate does not reach a steady and maximum value until half the final area has been attained.

Watson (1947) considers that under field conditions any ontogenetic drift in assimilation rate is small compared with the fluctuations caused by external environmental factors. From the limited data of the present experiments much the same conclusion could be reached; in two experiments the assimilation rate for the second period is higher than in the first and in one experiment there is no appreciable difference.

If it is postulated that the fluctuations in net assimilation rate in full daylight are related to a combination of light intensity and temperature, the question arises as to what extent shading alters the balance of these two factors and what are the differences in air and soil temperature beneath and outside the screens. In order to minimize such temperature differences the individual screens for the separate plots were kept to a minimum size (5 by 4 ft., or, in the case of exp. VI, 4 by 2 ft.) and they were set some 10 to 6 in. above the ground. In consequence there was a free circulation of air beneath and through the screens and any appreciable difference in the air temperature under and outside the screens should have been largely levelled out by wind and convection currents.

In addition, with a similar type of screening (single and double layers of butter muslin), it had already been shown (Blackman and Templeman, 1938) that a reduction in the light intensity to 0.4 of daylight produced only small changes in the soil temperature. At a depth of 4 in.—the depth at which the bluebell bulbs were planted—the soil temperature under the screens was on an average 0.3° C. higher at 9 a.m. and 0.5° C. lower at 5 p.m. Moreover, using mercury thermometers with the bulbs screened from direct insolation, no differences between the air temperatures under and outside the screens could be detected.

It is realized that this evidence is not critical enough to dismiss the possibility that *small* differences in air temperature did not exist, especially on still days and nights. Unfortunately, as Curtis (1936) has pointed out, the accurate determination of air temperature is particularly liable to interference by reflected radiation from nearby objects, such as the screens.

On the assumption that the temperature differences between the shaded and unshaded plants are small, and assuming that in full daylight the observed variations of 0.43 to 0.73 in the net assimilation rate are due to a combination of day length, light intensity, and temperature, it follows that a reduction in the light intensity by shading has the same logarithmically proportional effect on assimilation, irrespective of the seasonal differences in day length, light intensity, and temperature.

This somewhat surprising conclusion is supported by further investigations by the first author of the light factor in relation to the growth and assimilation rate of young sunflower (*Helianthus annuus*) plants. These investigations will be reported more fully in subsequent papers, but a few

of the results for the relationship between light intensity and net assimilation rate are shown in Fig. 13. It is clear that, just as with the bluebell, there is an exact linear relationship between the logarithm of light intensity and assimilation, although the individual experiments were carried out over the months of May to October and the rates in full daylight ranged from 0.295 to 0.763.

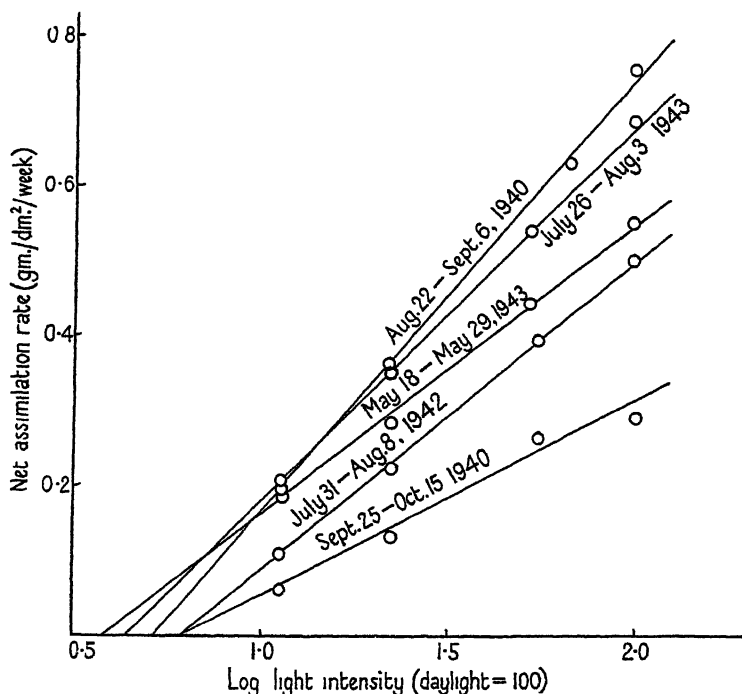


FIG. 13. The effects of varying light intensity on the net assimilation rate of sunflower (*Helianthus annuus*) seedlings at different periods in the summer.

The question therefore arises as to the factors which are involved in the reduction of the assimilation rate of shaded leaves. The possibility that the screens of perforated zinc altered the quality of the light can be dismissed, and even in the case of the butter muslin with its high light transmission and open weave the changes are likely to be negligible. The light effects therefore can be regarded as due to changes in intensity.

Since Fig. 3 demonstrates that the leaf-area per unit leaf-weight *increases* logarithmically with falling light intensity whereas Figs. 4-6 and 10 show that net assimilation rate *decreases* logarithmically with shading, it follows that leaf assimilation and the area : weight ratio are inversely correlated. On the other hand, the leaf-area : weight ratio largely reflects the changes in leaf structure associated with shading, and from this it follows in turn that the reduced assimilation rate may be linked with these modifications.

A reduction in assimilation rate of shaded leaves might therefore not only be associated with the decreased quantity of light reaching the leaves but also

with the proportion of light absorbed by the leaves. Evidence on the light absorption and transmission of shaded and unshaded leaves does not appear to have been critically examined, but Sauberer (1937) has investigated the light-transmitting capacity of young and old beech (*Fagus sylvatica*) leaves. These results showed that over a considerable range of wave-length thin leaves transmitted more light than leaves which had reached maturity. If immature leaves correspond to 'shade' leaves and mature leaves to 'sun' leaves, then there are grounds for concluding that light transmission may be one of the factors involved in the decreased assimilation rate of shaded leaves.

Apart, however, from a possible influence of reduced light intensity on the light absorption power of leaves, the modifications in leaf structure, stomatal number, &c., induced by shading might affect assimilation by altering the rate of gaseous exchange. Moreover, on the evidence of Däxer (1934), shade leaves may differ in their internal and metabolic factors.

From the present investigations no critical assessment of the possible importance of light transmission, gas exchange, or internal factors can be made. The results of experiment X (Table VI) do not suggest that these effects are large, since the net assimilation rate of plants previously subjected to sun and shade conditions did not differ when they were subsequently all shaded. The results can, however, by no means be regarded as conclusive since the experimental errors are high.

Finally there is the question of how far these experimental studies of net assimilation rate can be linked up with the conclusions reached in the woodland studies concerning the importance of the light factor in governing the distribution of the bluebell (Blackman and Rutter, 1946). Because of the linearity of the relationship between the logarithm of the light intensity and assimilation the extrapolation of the regressions given in Figs. 4-6 and 10 offer a new means of determining the compensation point with great accuracy; in fact with a greater precision than by any of the methods previously employed by other workers under field conditions.

As in the case of the assimilation rate, the compensation point for the bluebell shows some divergence between experiments. The values range from 0.078 to 0.165 of daylight, and it is clear from Fig. 12 that there is no correlation between the net assimilation rate in full daylight and the compensation point, since for the highest and lowest assimilation rates recorded (0.776 and 0.433) the compensation points are approximately the same—0.081 and 0.098 of daylight.

By combining the data for all the regressions of net assimilation rate on the logarithm of the light intensity a mean regression can be arrived at, and from this regression it can be calculated that the mean compensation point is 0.093 daylight, a figure which falls very closely into line with the 'light extinction points' deduced from the previous woodland investigations, i.e. the degree of shading at which bluebells cease to be a component of the ground flora.

Besides the net assimilation rates it is also possible to calculate the corre-

sponding rates of growth of the whole plant for all the experiments, and from these data to calculate a multiple regression relating light intensity and efficiency index. This regression is given in Fig. 14 together with the regression coupling light intensity and net assimilation rate. It is seen that the two curves are divergent especially at the higher light intensities. This divergence is due to the greater effect of shading in increasing the leaf-area per plant than in reducing the net assimilation rate per unit area of leaf. Nevertheless below

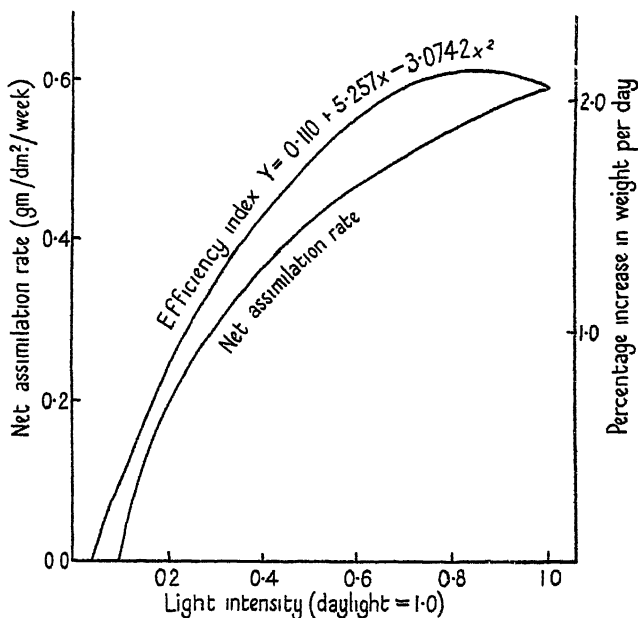


FIG. 14. The mean effect of the varying light intensity on (a) the growth-rate (*efficiency index*), and (b) the net assimilation rate of the bluebell: curves based on the data of experiments III, IV, V, VI, IX, and X.

0.6 daylight this compensatory mechanism does not prevent shading from lowering the growth-rate. Thus both these data and the previous woodland findings support the view that the bluebell is not an obligate shade plant, but is in fact sensitive to small reductions in the light intensity.

Finally experiments IX and X also have a bearing on the growth of the bluebell under woodland conditions. The field trials have shown that where the mean degree of shading over the season is made up of an initial phase of 'high' light (full daylight) and a subsequent 'low' light phase (0.08–0.22 of daylight) the plants make less growth than those subjected to the same *average* but constant degree of shading over the whole season. It has also been shown that these differences can mainly be ascribed to the larger leaf-area of those plants which have been shaded early, since the potential leaf expansion under shade conditions is greatest in the earlier stages of leaf development (Fig. 11).

These two sets of experimental conditions are analogous in the light differences in deciduous woodland where the overhead canopy 'breaks' early or late in the spring, e.g. larch as against oak. Where the expansion of the leaf canopy coincides with the initiation of growth by the bluebell, shading will limit the assimilation per unit leaf-area but will bring about a large increase in total leaf-area. On the other hand, where there is a pronounced high light phase, due to late leaf expansion of the trees, the initial assimilation rates will be high because of the high light intensity; but the level of light intensity in turn will restrict the leaf-area per plant during the high light phase while during the subsequent low light phase the extent to which the leaves can increase in area is now restricted.

### SUMMARY

In previous papers of this series it has been demonstrated that where in deciduous woodland the overhead canopy is closed the distribution of the bluebell is largely determined by variations in the degree of shading. A subsequent analysis of the environmental factors controlling seasonal growth showed that while the bluebell is comparatively insensitive to variations in the levels of nitrogen, phosphorus, and potassium supply it is markedly affected by even small reduction in light intensity. Below an intensity of half daylight the rate of growth is exclusively determined by the light factor.

The present paper provides a further study of how the light and mineral nutrient supply factors operate. Data are available from six multifactorial experiments, involving up to twenty-four combinations of light intensity and mineral nutrition. In each experiment random samples from each plot were from time to time withdrawn during the season and on each occasion the weights of leaf, inflorescence, and bulb were determined, and measurements of the leaf-area made.

It has been established that whereas shading down to a level of 0.11 daylight causes little variation in the total leaf-weight per plant, it nevertheless brings about a progressive increase in total leaf-area. This increase in leaf-area is reflected in the change in the ratio of unit leaf-area to unit leaf-weight. Over the range of full daylight to 0.11 daylight *this ratio bears an inverse linear relation to the logarithm of light intensity.*

For nine sets of observations, spread over three seasons, the net assimilation rate in full daylight ranged from 0.43 to 0.78 gm. per sq. dm. per week, with a mean value of 0.59. There was no evidence that these fluctuations were related to an ontogenetic drift in assimilation during the period of active growth in April and May.

In every instance it has been found that the *net assimilation rate is directly proportional to the logarithm of the light intensity.* From this it can be inferred that even in full daylight the net assimilation rate is controlled by the duration and intensity of daylight ruling in the spring.

Because of this exact correlation between net assimilation rate and the

logarithm of the light intensity it is possible, by extrapolation of fitted regression equations, to calculate with an entirely new precision the 'compensation point' under field conditions. The values obtained for the degree of shading where the net assimilation rate is reduced to zero ranged from 0.08 to 0.17 daylight, with a mean value of 0.089. Differences in the compensation point values are not related to the level of assimilation in full daylight.

The net assimilation rate is unaffected by variations in the supply of nitrogen, phosphorus, or potassium. Where the rate of growth is affected by nutrient supply the increased rate is in the main due to the increases in leaf-area resulting from additional nutrients. Significant but small increases in leaf-area were recorded for nitrogen in three experiments, for phosphorus in one experiment, but there were no significant effects in the case of potassium.

Bluebells which have been grown for an initial period (April) under reduced light intensity subsequently make better growth under shade conditions (in May) than plants which have received full daylight over the initial period. This 'conditioning' effect of the initial low light phase can be ascribed to the increase in leaf area induced by preliminary shading; over the subsequent shade period such plants have a higher relative leaf-area than those subjected to an initial high light phase.

The effects of shading on the growth rate of the bluebell involve the compensatory effects of shading on net assimilation rate and total leaf-area. Between full daylight and 0.7 daylight the rate of growth remains approximately constant, since the fall in assimilation rate is compensated for by the increase leaf-area; below 0.6 of daylight the reduction in net assimilation rate is not balanced by a further increase in leaf-area.

It is concluded that the results of the present experiments confirm and amplify the conclusions reached in the previous field and woodland studies. For example, the light values for the 'compensation point' are in good agreement with the 'extinction points' obtained in the woodland investigation, i.e. the degree of shading where the bluebell ceases to be a component of the ground flora.

It is also concluded that variations in the light intensity may affect not only the net assimilation rate through a reduction in the light energy reaching the leaves, but may also alter the assimilation by the effects of shading in modifying leaf structure and, possibly, also the internal metabolic factors. The complex of factors involved is not confined to the bluebell. Supporting evidence is given that *the same linear relationship between net assimilation rate and the logarithm of the light intensity also holds for the sunflower (*Helianthus annuus*)*.

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# The Growth-inhibitory Activity of the Sulphonamides and Plant Growth-substances, and the Effects thereon of *p*-Aminobenzoic acid

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With two Figures in the Text

## INTRODUCTION

VERY soon after the work of Tréfouël, Tréfouël, Nitti, and Bovet (1935) on the effectiveness of sulphanilamide as a bacteriostatic agent, investigations were extended to its action on the growth and development of moulds (*Aspergillus* spp.) and higher plants (Cress, *Lepidium sativum*) (Fourneau, Tréfouël, Tréfouël, Nitti, and Bovet (1936)). In the latter case, an inhibition of development was obtained by a concentration of 1/1,000 of sulphanilamide. Two years later Grace (1938) published conclusions from experiments on yeast, cuttings of higher plants (tomato), and seeds, in which he claims to have shown that sulphanilamide in low concentrations 'acts like the recognised plant hormone chemicals' and '... does possess growth-promoting properties'. Higher concentrations are growth inhibitory. This publication, however, is considerably lacking in experimental details, and its value correspondingly difficult to assess. Stimulated presumably by the discovery of Woods (1940) that the bacteriostatic action of sulphanilamide can be neutralized completely by very small amounts of *p*-aminobenzoic acid, Mangenot and Carpentier (1941 and 1941a) investigated the action of sulphanilamide, *p*-aminobenzoic acid, and the plant growth hormone,  $\beta$ -indoleacetic acid, on the growth of roots of *Pisum sativum* and *Lupinus* sp. They found that both  $\beta$ -indoleacetic acid (M/10,000 and M/100,000) and *p*-aminobenzoic acid (M/1,000 and M/10,000) stimulated the growth of lateral roots, but on the other hand sulphanilamide (M/100, M/1,000, and M/10,000) suppressed longitudinal root growth. *p*-Aminobenzoic acid was also found to neutralize these 'anti-rhizogenous' properties of sulphanilamide, but to augment the 'rhizogenous' properties of  $\beta$ -indoleacetic acid. Similar results were obtained by Wiedling (1943), who found that a concentration of 3 parts in 10,000 of sulphanilamide was sufficient to cause complete inhibition of the longitudinal root growth of *Pisum sativum* grown on agar under sterile conditions. The antagonistic effect of *p*-aminobenzoic acid was also confirmed both with sulphanilamide and other inhibiting sulphonamides, such as sulphapyrimidine. He suggests that these facts fit in with the Woods-Fildes

theory (Woods and Fildes, 1940), and that *p*-aminobenzoic acid is also an essential metabolite in plants. Similar phenomena were reported by Bonner (1942) from experiments on excised tomato roots grown *in vitro*. Finally Macht and Kehoe (1943), working with *Lupinus albus*, observed stimulation of growth with sulphanilamide at concentrations less than 5 parts per million and inhibition at 40 parts per million and over. *p*-Aminobenzoic acid gave stimulations at concentrations less than 1 part per million and inhibitions at greater concentrations. A mixture of the two compounds exerted a more toxic influence than could be obtained by adding the effects of the individual constituents, a result in direct opposition to those obtained by previous workers.

It is obvious from the foregoing summary that both sulphanilamide and *p*-aminobenzoic acid have marked physiological actions on plant growth, particularly that of roots, but the precise nature of the plant response and the effective concentrations of the drugs seem somewhat obscure. In addition, the antagonistic action of *p*-aminobenzoic acid on sulphanilamide inhibition seems open to some doubt. In view of this rather unsatisfactory state of our knowledge, it was decided to carry out experiments on root growth in a range of concentrations of various sulphonamides, and to compare results with those obtained with the synthetic plant growth hormones, on which much work has already been done (Nutman, Thornton, and Quastel, 1945; Audus and Quastel, 1947), thus checking the claims of Grace (1938). In addition, experiments were designed to test the possible antagonistic effect of *p*-aminobenzoic acid over a range of concentrations, both with the sulphonamides under consideration and also with some of the synthetic plant growth-substances, e.g. 2-4: dichlorophenoxyacetic acid (to be referred to as 2-4 : D.). In addition to clearing up the confusion in the literature as outlined above, it was hoped to throw some light not only on the mode of action of the sulphonamides on plants, but also possibly on that of the plant growth hormones themselves.

#### EXPERIMENTAL METHODS

Cress (*Lepidium sativum*) was chosen as the experimental material, since much is already known concerning its reaction to the more common synthetic growth substances, e.g. 2-4:D. Seedlings were grown in  $\frac{3}{4}$ -in. test-tubes in water-culture solutions under rigidly sterile conditions and by the technique described in a previous paper (Audus and Quastel, 1947). In each experiment the effect of a range of concentrations of neutralized *p*-aminobenzoic acid and of either a sulphonamide or a plant hormone were investigated. All possible combinations of the various concentrations of the two components were set up. The final pH of all solution mixtures was about 6.8. Twenty seeds per test-tube were sown and, after 7 days' growth in artificial light at 20° C., seedlings were removed from the tubes, the roots cut off with a sharp scalpel, dried rapidly on filter-paper, and weighed to the nearest 0.1 mg. From these weights the root growth per seed germinated (i.e. total weight (mg.) of roots per tube divided by the number of seedlings) was calculated, and this value, expressed as a percentage of that in the control tube (normal growth), has been

called the Root Growth Index (R.G.I.). This gives a direct indication of the reduction in total root growth brought about by the inhibitor, and it eliminates any variations due to the different germinative capacity of the various samples.

#### RESULTS WITH SULPHONAMIDES

The results of these experiments appear in Fig. 1, where they are plotted as three dimensional diagrams, the three variables, root growth index, and concentrations of the two substances under consideration, being plotted along three mutually perpendicular axes. Thus the base of each diagram is a grid constituted by the various combinations of concentrations of the two compounds concerned, whereas the height of each ringed point above the base is a measure of the R.G.I. for the two relevant concentrations.

The four diagrams on the left of the figure are for results with four of the sulphonamides: sulphanilamide, sulphapyridine, sulphathiazole, and sulphaguanidine. In each of these graphs values of R.G.I. in the absence of the sulphonamides and in a range of concentrations of *p*-aminobenzoic acid are calculated from a number of parallel growth experiments. The thick vertical lines are drawn about a mid-point of the mean for a number of values, the range on either side of this mean being equal to the standard error of that mean. The number of measurements in the sample is indicated by the figure above each line. For observations in *p*-aminobenzoate-sulphonamide mixtures individual observations have been plotted. Each value is based on the measurement of approximately 20 roots. From these four diagrams, which form a very uniform series, the following conclusions can be drawn:

(a) All four sulphonamides, when acting alone, produce marked inhibition of root growth, even at concentrations as low as 10 parts per million, but even at the highest concentration of 1,000 parts per million it was not inhibited completely. Sulphanilamide and sulphapyridine seem rather more effective than either sulphathiazole or sulphaguanidine.

(b) There is an indication that sulphanilamide and sulphaguanidine at a concentration of 1 part per million produce some slight stimulation of root growth, but the difference (*c.* 18 per cent.) is of the order of the experimental error.

(c) *p*-Aminobenzoic acid when acting alone produces a slight but significant inhibition of root growth at a concentration of 10 parts per million and one of 55 per cent. at 1,000 parts per million.

(d) *p*-Aminobenzoic acid has a definite neutralizing effect on the inhibitive action of the sulphonamides. This effect is most clearly marked at concentrations of sulphonamides of 10 and 100 parts per million. At each of these concentrations root growth is increased, by increase of *p*-aminobenzoic acid concentration, up to a maximum equal to or approaching 100 per cent. of control (*i.e.* complete neutralization of inhibition) when the two concentrations (*i.e.* of *p*-aminobenzoic acid and sulphonamide) are equal. Further increase in the concentration of *p*-aminobenzoic acid results in a decrease of the root growth index as its own inhibitive effect becomes apparent. Thus at *p*-aminobenzoic acid concentrations of 1,000 parts per million there is no significant

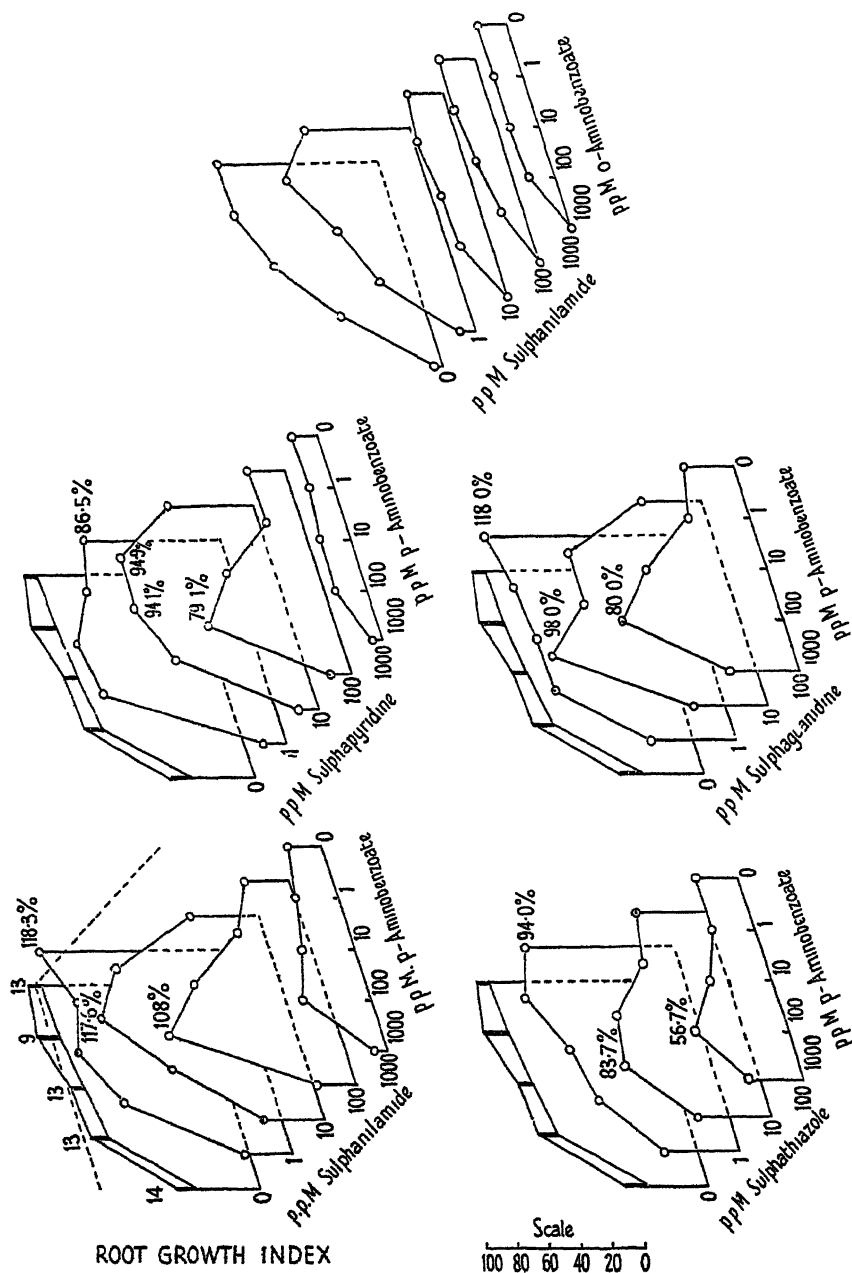


FIG. 1. The interaction of *p*-aminobenzoic acid with four sulphonamides (sulphanilamide, sulphapyridine, sulphathiazole, and sulphaguanidine) and also of ortho-aminobenzoic acid with sulphanilamide on the root growth of cress (*Lepidium sativum*).

effect of any of the sulphonamides, and the low growth is probably due entirely to the toxic action of these high *p*-aminobenzoic acid concentrations.

Results of experiments on sulphanilamide and ortho-aminobenzoic acid strongly suggest that the antagonistic action of *p*-aminobenzoic acid is a specific one. These results appear in the right-hand diagram of the figure. Here it will be seen that ortho-aminobenzoic acid is somewhat more toxic than the para-compound when acting alone. In addition there is no evidence of any significant antagonistic action on sulphanilamide inhibition of root growth.

#### RESULTS WITH PLANT GROWTH-SUBSTANCES

The results of experiments with the synthetic plant growth-substance 2-4: dichlorophenoxyacetic acid and with *p*-aminobenzoic acid are seen in the diagram at the top left-hand corner of Fig. 2. Here figures for a number of experiments are included, each ring representing a value from the growth of approximately 20 seedlings compared with that of the controls. The results for *p*-aminobenzoic acid alone are plotted as in Fig. 1. The continuous lines forming the tops of each of the other vertical planes connect the means of the relevant groups of observed points. The vertical range of points in each group therefore gives an idea of the biological variation inherent in this technique.

From this figure it would seem that *p*-aminobenzoic acid has a definite antagonistic action on the activity of 2-4:D. at low concentrations. A consideration of the values for 0.1 part per million of 2-4:D. shows that *p*-aminobenzoic acid at concentrations of 10 and 100 parts per million brings about a small but significant increase in the root growth index as compared with the values in the absence of *p*-aminobenzoic acid. In addition, values of the root growth index for increasing *p*-aminobenzoic acid concentrations in presence of 0.1 part per million 2-4:D. and the corresponding values in the absence of 2-4:D. gradually approach equality at a *p*-aminobenzoic-acid concentration of 1,000 parts per million. Even at 1 part per million of 2-4:D. the values of the root growth index for high concentrations of *p*-aminobenzoic acid are much greater than would be expected if the two inhibitive effects were additive, suggesting again some degree of mutual antagonism. It must be noted that, in contrast to the results with the sulphonamides, neutralization approaches completion only when the concentration of *p*-aminobenzoic acid is of the order of 10,000 times that of 2-4:D.

Experiments have also been carried out on three other growth substances, viz. 4-chloro-2-methyl-phenoxyacetic acid,  $\beta$ -naphthoxyacetic acid, and  $\beta$ -indoleacetic acid, and results from these also appear in Fig. 2. In the case of 4-chloro-2-methyl-phenoxyacetic acid a definite indication of antagonistic action of *p*-aminobenzoic acid is seen in a concentration of 1,000 parts per million. There is no evidence of such an action at the lower concentrations of *p*-aminobenzoic acid as in the case of 2-4:D. With  $\beta$ -naphthoxyacetic acid and  $\beta$ -indoleacetic acid the action is smaller still, although there is distinct evidence that some antagonism is taking place. Thus at a

concentration of  $\beta$ -indoleacetic acid of 0.1 part per million there is no significant effect of *p*-aminobenzoic acid on the root growth index. This may be interpreted as an antagonism of *p*-aminobenzoic acid inhibition by  $\beta$ -indoleacetic acid or vice versa. The practical effect is the same, i.e.,

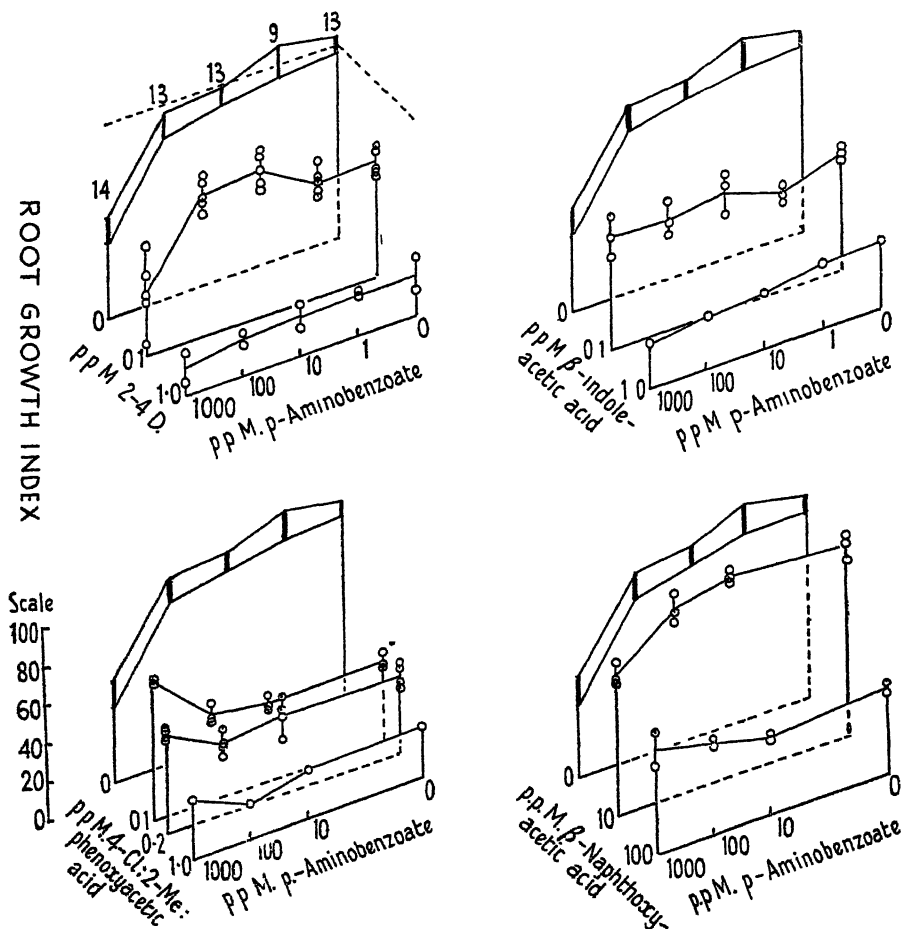


FIG. 2. The interaction of *p*-aminobenzoic acid with four plant growth hormones 2-4:dichloro-phenoxyacetic acid, 4-Cl:2-Me:phenoxyacetic acid,  $\beta$ -naphthoxyacetic acid (and  $\beta$ -indoleacetic acid) on the root-growth of cress (*Lepidium sativum*).

root growth in *p*-aminobenzoic acid concentrations of 1,000 parts per million is the same whether  $\beta$ -indoleacetic acid at low concentrations is present or not. This also holds for  $\beta$ -naphthoxyacetic acid at 100 parts per million ( $\beta$ -naphthoxyacetic acid is about 1/500 as effective on cress as 2-4:D. or  $\beta$ -indoleacetic acid).

#### CONCLUSIONS

The results of experiments here described support the findings of previous workers that the sulphonamides exert a definite inhibitory effect on root

growth at concentrations as low as 10 parts per million. Stimulation of root growth at lower concentrations, as claimed by Grace (1938), was indicated by results in the lower concentration of 1 part per million of sulphanilamide, and sulphaguanidine, but not with the other two drugs. *p*-Aminobenzoic acid is toxic, even at a concentration of 10 parts per million, while at 1,000 parts per million the inhibition reaches 55 per cent. In a few cases some evidence of the so-called 'rhizogenous' action of *p*-aminobenzoic acid was observed in these high concentrations, when a few small lateral roots appeared towards the base of the main root. No such lateral roots were ever observed during the normal growth of cress roots over the experimental period (7-10 days).

Turning to the interaction of the sulphonamides and *p*-aminobenzoic acid, the findings of Bonner (1942) and Wiedling (1943) have been verified. In the case of the four sulphonamides investigated results indicate that the antagonism of inhibition was complete when the concentration of *p*-aminobenzoic acid approximated to that of the sulphonamide. No significant difference in the behaviour of the four drugs is discernible from the results using this particular technique. The lack of any antagonistic action of ortho-amino-benzoic acid suggests that, as in the case of the bacteriostatic action of the sulphonamides, the action of *p*-aminobenzoic acid is a specific one.

One of the most important features of these results is that, for complete removal of inhibition, the concentration of *p*-aminobenzoic acid is found to be of the same order as that of the sulphonamide. This result is in strong contrast to the results on the inhibition of bacterial growth, where complete neutralization is obtained by *p*-aminobenzoic acid at very low concentrations of 1/50th to 1/4,000th that of the sulphonamide (Wyss, Grubaugh, and Schmelker, 1942). This last concentration is roughly proportional to, and of the same order as, that of the ionized fraction of the total sulphonamide concentration (Fox and Rose, 1942). It seems unlikely, therefore, that in the inhibition of root growth, sulphonamides are acting in the same way as in the bacterial cell. In the latter case an opinion has been expressed, in a very comprehensive review by Henry (1944), that '... sulphonamides inhibit cell division by interfering with a specific fraction of the oxidative metabolism of the cell'. *p*-Aminobenzoic acid antagonizes this action competitively, although the exact mechanism of this antagonism still remains a subject for controversy (see Henry, 1944). In the plant root, however, the competitive efficiency of the *p*-aminobenzoic acid molecule is very much smaller, suggesting that this competition may involve a completely different metabolic system.

In addition to these findings, significant antagonistic action by *p*-aminobenzoic acid has been demonstrated in the case of root-growth inhibition by the synthetic growth hormones. A concentration, however, of *p*-aminobenzoic acid of about 10,000 times that of the inhibiting hormone is required for any marked degree of neutralization. It seems probable, therefore, that the site of action in the root cell of the growth-inhibiting sulphonamides differs from that involved in plant growth hormone inhibition.

## SUMMARY

The four sulphonamides, sulphanilamide, sulphapyridine, sulphathiazole, and sulphaguanidine, have been shown to inhibit root growth of cress at concentrations as low as 10 parts per million; *p*-aminobenzoic acid antagonizes this inhibitory action, neutralization being complete when the concentration ratio *p*-aminobenzoic acid concentration sulphonamide approximates to unity.

*p*-Aminobenzoic acid also shows some slight antagonism to the root-growth-inhibitory action of the plant growth-substances, 2-4: dichlorophenoxyacetic acid, 2-chloro-4-methyl-phenoxyacetic acid,  $\beta$ -naphthoxyacetic acid, and  $\beta$ -indoleacetic acid. Here, however, for any marked degree of neutralization, the concentration ratio *p*-aminobenzoic acid/growth substance must be of the order of 10,000.

It is concluded from these ratios that the phenomenon of root-growth inhibition by sulphonamides is quite distinct from that of sulphonamide bacteriostasis, and also from that of root-growth inhibition by plant growth-substances.

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# Studies in Stomatal Behaviour

## I. Stomatal Movement induced by Heat-shock Stimuli, and the Transmission of such Stimuli across the Leaves of *Pelargonium zonale*<sup>1</sup>

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With eight Figures in the Text

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### INTRODUCTION

THERE does not seem to be any previous work on the transmission of heat-shock across leaves using stomatal resistance as an index; extensive investigations have of course been made into the mechanisms of shock-transmission in the various sensitive plants, but the effect on stomata does not appear to have received detailed attention. Isolated observations on shock-induced stomatal movements have been made by a number of workers, who have not, however, been concerned with the possibility of the transmission across a leaf of the effects in question.

The experiments described were carried out on *Pelargonium zonale* var. *Paul Crampel*, specimens of which were obtained from several sources. This plant exhibits two well-marked and distinct shock effects, according to whether the stimulus is produced by mechanical disturbance or by heat; the characteristics of the effects resulting from mechanical shock will be the subject of a later communication, the present research dealing entirely with the phenomena resulting from a heat-shock applied by burning the edge of a leaf.

### APPARATUS AND METHODS

#### *Apparatus*

The resistance porometer (Gregory and Pearse, 1934) has been used throughout. The porometer cups were simple brass cups, each with a glycerol-

<sup>1</sup> Part of a thesis approved for the degree of Ph.D. in the University of London, 1940.

gelatin ring (35 per cent. gelatin in equal parts glycerol and water); the ring was coated with a luting-wax composed of beeswax and vaseline, and the leaf was clamped between the ring and a glass plate. Since for the majority of the experiments it was necessary to attach two cups to the same leaf, two cups of this type were fixed into a single ebonite holder with their centres 5 cm. apart;

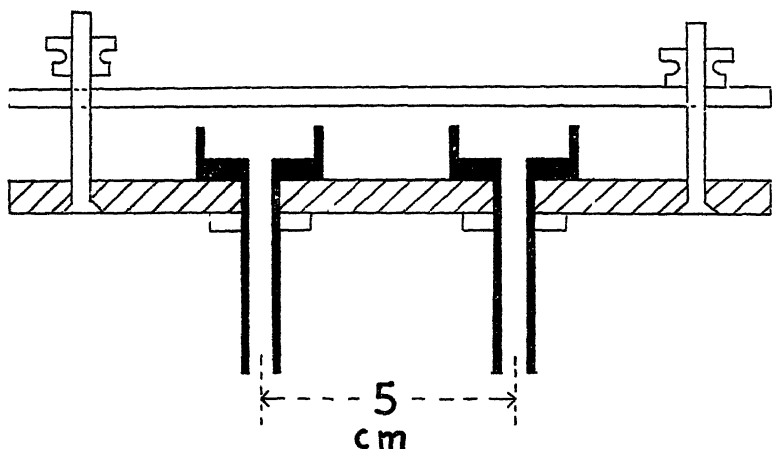


FIG. 1. Diagram of ebonite holder for porometer cups.

a diagram of this holder, which was made for the writer by Mr. W. Shaw, is shown in Fig. 1. The complete apparatus is shown diagrammatically in Fig. 2; the general arrangement is conventional, and only minor points require mention. It will be observed that the connexions (of pressure-tubing) between the cups and the rest of the apparatus could be closed, and that the cups could be put into communication with each other and with the outside air. The capillary resistances  $R_1A$  and  $R_1B$  were of thermometer tubing (each  $7^\circ$  of a  $100^\circ$  C. thermometer), and were equal within the limits of error of the experiments;  $MA$  and  $MB$ , the manometers, contained dilute aqueous methylene blue. The constant-pressure aspirator calls for no explanation apart from the use of the three taps; at the start of an experiment  $T_1$  was closed and  $T_2$  opened and at the close of an experiment this procedure was reversed; when it became necessary to refill the aspirator,  $T_1$  was opened,  $T_2$  closed, and  $T_3$  turned to its alternative position.

The stomatal resistance at any time is given by

$$R_2 = \frac{P_2}{P_1 - P_2} \cdot R_1, \quad (1)$$

where  $R_2$  = stomatal resistance,  $R_1$  = fixed capillary resistance,  $P_2$  = manometer pressure, and  $P_1$  = constant head of water. Since only comparative results are required, no attempt has been made to reduce the values thus obtained to 'Pearse units' or actual rate of flow.

### Methods

The two cups were affixed to the selected leaf, care being taken to ensure that each cup was *between* two of the main veins, and the plant was allowed to remain in light until the stomata recovered and reopened; about 2 hours was normally allowed. (In a few experiments one cup only was used; in all experiments the leaf was attached to the plant.) The edge of the leaf was burnt

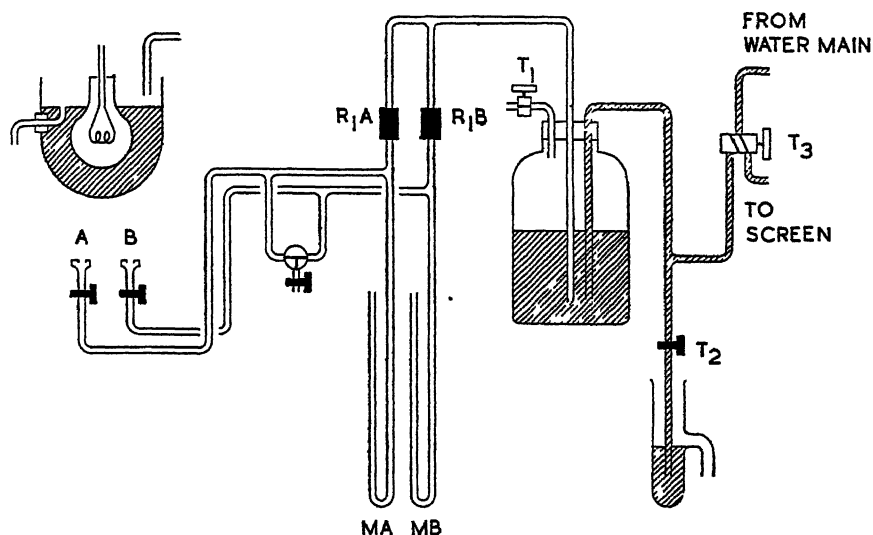


FIG. 2. Diagram of complete apparatus.

with a fine inch-long gas-jet for about 2 seconds and the manometers were read at 1-minute intervals thereafter. At the conclusion of the experiment the leaf was removed from the apparatus and from the plant, laid on a sheet of paper, and the outline traced; the position of the burn was noted, and the position of the porometer cups marked by piercing the leaf with a pencil in the middle of the luting-wax ring, which remained on the leaf; finally, the main veins were drawn in approximately by eye. In this manner a record of the topography of the leaf was obtained to an accuracy which, though not great, is considered adequate for the purpose of these experiments.

## EXPERIMENTAL RESULTS

### I. The Independence of Leaf-sectors bounded by Main Veins

The experimental method described above is valid only if, under normal conditions, the two groups of stomata being investigated react to external stimuli independently of each other. Furthermore, it is necessary that the closure of one group of stomata should be completely without effect on the flow of air through the other group (cf. the work of Darwin (1916) on *Prunus laurocerasus*, in which the effect on porometer readings of vaselining part of the leaf was investigated). Heath (1941) has pointed out that the leaf of *Pelargonium* is homobaric so far as any individual sector enclosed by two

main veins is concerned; but that 'it is probable that the main veins provide an almost complete barrier to gas movement either by viscous or diffusive flow'. To test this view a number of simple experiments were performed in which the two areas concerned were subjected to different conditions by alternate shading and illumination. The behaviour of the stomata in such circumstances is shown (Expt. 1) in Fig. 3, in which, since high stomatal resistances are difficult both to measure accurately and to represent adequately on a graph, the ordinates represent manometer pressures.

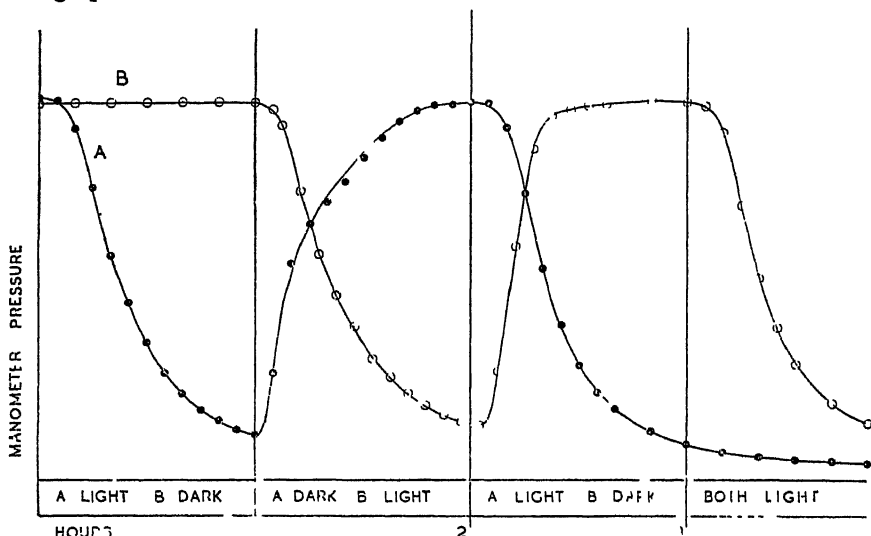


FIG. 3. Stomatal behaviour in Experiment 1 with two areas A and B alternately illuminated and shaded.

It appears clear from this diagram that the two groups of stomata can be regarded as reacting completely independently of each other. However, results have recently been obtained which show that under certain conditions the porometer may draw air across the main veins, thereby producing an apparent dependence of the two groups of stomata. What precisely these conditions are is under investigation; but the phenomenon is at least not to be expected when the stomata, as has been the case in the experiments to be described, are not far from the open position; for in such circumstances virtually all the air is drawn straight through the leaf from the stomata in the upper epidermis over the porometer cup (Heath, 1941). The effect has certainly not been observed in the course of the present work, and it will therefore be ignored in this communication.

## II. Transmission in Intact Leaves

### (a) Results

The results of four experiments are set out in Table I.  $P_2(A)$  and  $P_2(B)$  are the manometer pressures corresponding to the cups respectively nearer to, and farther from, the point of burning; the columns headed  $S(A)$  and  $S(B)$

are explained below. Diagrams of the four leaves concerned are given in Fig. 4.

TABLE I

	Time (min.).	$P_2(A)$ .	$S(A)$ .	$P_2(B)$ .	$S(B)$ .
Expt. 2 (Fig. 4 (a))	0	7.30	—	8.34	—
	1	7.30	0.00	8.34	0.00
	2	7.30	0.00	8.34	0.00
	3	7.46	0.22	8.40	0.08
	4	7.86	0.57	8.42	0.03
	5	8.30	0.62	8.42	0.00
	6	—	—	8.42	0.00
	7	—	—	8.42	0.00
	8	—	—	8.52	0.13
	9	—	—	8.82	0.41
	10	—	—	9.24	0.59
	11	—	—	9.56	0.45
Expt. 3 (Fig. 4 (b))	0	2.27	—	1.88	—
	3	2.31	0.04	1.88	0.00
	5	2.39	0.11	1.90	0.03
	6	2.69	0.84	1.92	0.07
	7	3.29	1.54	1.92	0.00
	8	3.95	1.48	1.92	0.00
	9	—	—	1.94	0.07
	10	—	—	2.08	0.45
	11	—	—	2.42	1.08
	12	—	—	2.92	1.42
	13	—	—	3.54	1.52
Expt. 4 (Fig. 4 (c))	0	1.35	—	2.17	—
	3	1.35	0.00	2.17	0.00
	4	1.47	0.74	2.17	0.00
	5	1.85	2.21	2.17	0.00
	6	2.43	2.82	2.17	0.00
	7	—	—	2.17	0.00
	8	—	—	2.37	0.83
	9	—	—	2.57	0.77
	10	—	—	2.83	0.95
Expt. 5 (Fig. 4 (d))	0	2.73	—	2.23	—
	1	2.91	0.62	2.31	0.32
	2	2.93	0.06	2.33	0.08
	3	2.97	0.13	2.37	0.15
	4	3.07	0.32	2.39	0.08
	5	3.33	0.83	2.39	0.00
	6	3.83	1.52	2.39	0.00
	7	4.29	1.30	2.39	0.00
	8	—	—	2.43	0.15
	9	—	—	2.57	0.52
	10	—	—	2.67	0.36

### (b) Treatment of results

(i) *Relative change.* We require to know the change in stomatal resistance that has occurred at the end of each minute. However, the absolute value of this change is not an entirely reliable index, partly because the extent of movement is likely to be affected by the average stomatal aperture at which the experiments are conducted, and partly because the resistance porometer is

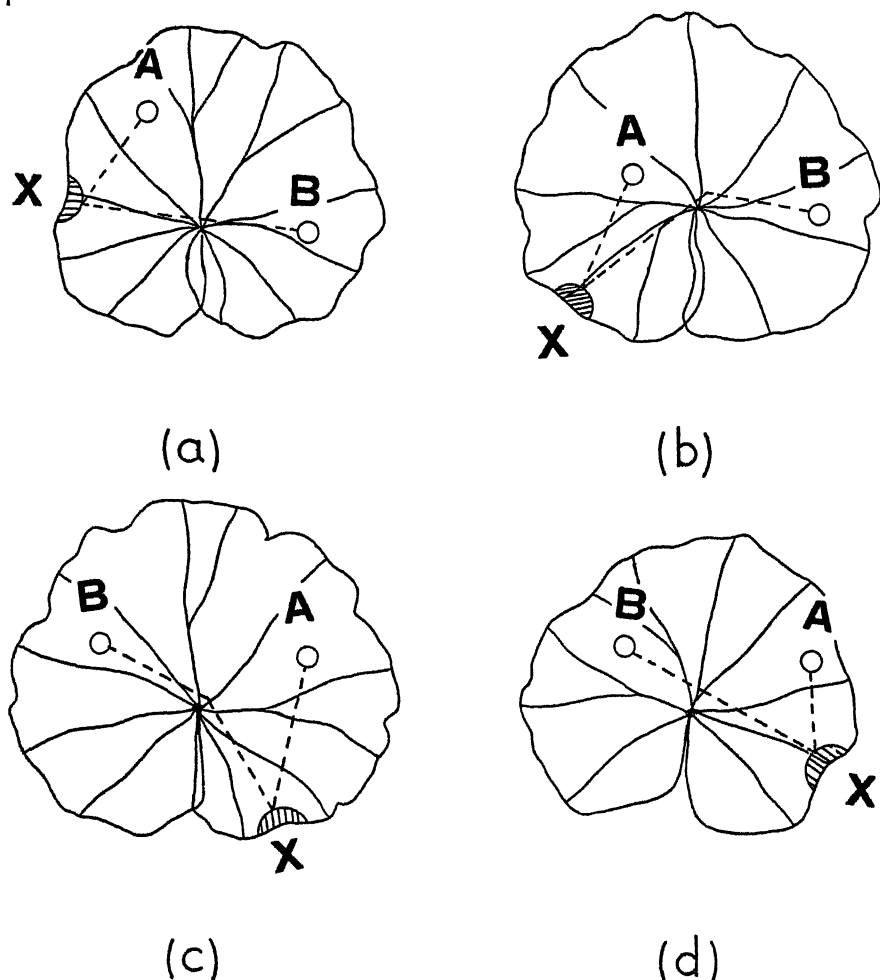


FIG. 4. Diagrams of the four leaves used in Experiments 2-5. See Table I.

not of constant sensitivity over the whole range of stomatal aperture. For this reason it has been considered desirable to calculate the *percentage increase* in stomatal resistance that has occurred during each minute; i.e. if the resistances at the beginning and end of the minute are  $R_a$  and  $R_b$  respectively, the quantity required is  $(100/R_a)(R_b - R_a)$ . It is convenient to work this out directly from the manometer pressures; if  $P_a$  and  $P_b$  are the manometer pressures corresponding respectively to  $R_a$  and  $R_b$ , and  $P_1$  has its usual significance, then substituting in equation (1) above gives

$$P_1 \left[ \frac{100(P_b - P_a)}{P_a(P_1 - P_a)} \right]. \quad (2)$$

We shall for convenience refer to the expression within square brackets as the 'relative change' and denote it by  $S$ ; this is the quantity set out in Table I in the columns headed  $S(A)$  and  $S(B)$ , corresponding to porometer cups  $A$  and  $B$ .

(ii) *Initial effects.* In experiments 2 and 3 a slight movement of  $B$  is discernible immediately  $A$  begins to move. This is, however, only temporary;  $S(B)$  does not reach a value comparable with that of  $S(A)$  and quickly dies away to zero, what is presumably the genuine shock effect beginning later. It is possible that this effect is brought about simply by a draught of hot air over the leaf from the small flame used; the capriciousness of the phenomenon—it is absent in Expt. 4 and has affected both cups in Expt. 5—is in keeping with this hypothesis, which will be further investigated. Occasionally, on the other hand, the first discernible effect of the shock has been a transitory slight *fall* in resistance at the nearer cup; but the behaviour of leaves is so variable, and the fall so slight, that the reality of the phenomenon cannot be said to be established. For the purpose of the present research all initial movements of either type will be neglected in calculation.

(iii) *Threshold value of  $S$ .* There are two possible methods of comparing the effects recorded at the two cups. First, an arbitrary value of  $S$  may be taken, so that when this is attained the shock effect may be considered to start. This will ensure that only effects comparable in magnitude are compared. It is not certain, however, that this is desirable; it is conceivable that the more distant area will receive a diminished shock effect. Alternatively, the shock may be said to begin at the first measurable increase in manometer pressure, initial effects being ignored. Calculations will be carried out in both ways, using an arbitrary threshold in the first case of  $S = 0.50$ . Since the relative change calculated from the manometer readings at the end of any one minute presumably represents what has occurred during that minute, it will be assumed that, if the appropriate shock effect occurs  $t$  minutes after the beginning of the experiment, the shock reaches the area under observation at  $(t-1)$  minutes after the beginning. Furthermore, if owing to a deficiency of observations  $S_n$  represents an effect over a period of  $n$  minutes it will be assumed that the relative change per minute ( $S_1$ ) has been constant over that period. It can then easily be shown that

$$(1+s_1)^n = (1+s_n), \quad (3)$$

where  $S = 100s$ ; and that, for the orders of magnitude of  $S$  found in the experiment a good approximation to this equation is given by  $S_1 = S_n/n$ . This relation was used to obtain the first two values of  $S$  in Table I, Expt. 3.

(iv) *Measurement of distance.* The distance from the burn to each of the porometer cups has been measured on the diagrams, the shortest practicable path having been measured in each case. These paths are shown as dotted lines in Fig. 4.

### (c) Preliminary consideration of results

The observations are summarized in Table II, in which the symbols in column 1 have the following meaning:

$x_a$  distance (cm.) from the edge of the burn to the centre of the nearer porometer cup.

$x_b$  corresponding distance to the farther porometer cup.

$t_a$  time in minutes (less 1 minute) at which any appreciable effect is observed at the nearer porometer cup (neglecting initial effects).

$t_b$  corresponding time for the farther cup.

$T_a$  time in minutes (less 1 minute) at which the relative change exceeds 0.50 at the nearer porometer cup.

$T_b$  corresponding time for the farther cup.

The two sets of distance/time ratios are included in the table, and it is clear that the ratio of distance to threshold time is the more consistent in three of the four cases. The value of the ratio is not markedly different for different leaves, the grand mean of all eight  $x/T$  values being 0.75 cm. per minute. Consideration of the significance of these results will be deferred until the general discussion of the results.

#### (d) Later effects

Consider Fig. 5. This represents the later behaviour of the stomata in two experiments, 6 and 7, which are similar to the foregoing; the time-scale is necessarily somewhat compressed, and the early parts of the graphs are not accurate. These curves show three features of interest: (i) both cups reach their maximum closure at the same time; (ii) the degree of closure is of the same order for both cups, and does not even remotely approach that attained in darkness; (iii) after maximal closure the resistance falls irregularly; but it falls faster, more regularly, and farther, in the area which is farther away from the burn. The tendency of the nearer stomata to oscillate has been noted in practically all these experiments.

TABLE II

1	Expt. 2.	Expt. 3.	Expt. 4.	Expt. 5.
$x_a$	2.5	3.0	3.6	2.0
$x_b$	6.0	6.6	6.2	5.2
$t_a$	2	2	3	1
$t_b$	7	8	7	7
$T_a$	3	5	3	4
$T_b$	9	10	7	8
$x_a/t_a$	1.25	1.50	1.20	2.00
$x_b/t_b$	0.86	0.83	0.89	0.74
$x_a/T_a$	0.83	0.60	1.20	0.50
$x_b/T_b$	0.67	0.66	0.89	0.65

The third of these observations suggests that there may be some slight lasting damage to the stomata. Such damage is not easy to demonstrate; but some indication may be obtained by inducing normal light and dark movement in a leaf which has been subjected to the burning treatment. The results thus obtained have not been sufficiently conclusive to justify detailed citation, but in those cases so far examined there appears to be a distinct tendency for the stomata nearer the burn to close less, and subsequently to open less, than those farther away. Moreover, the shape of the curves produced by the nearer

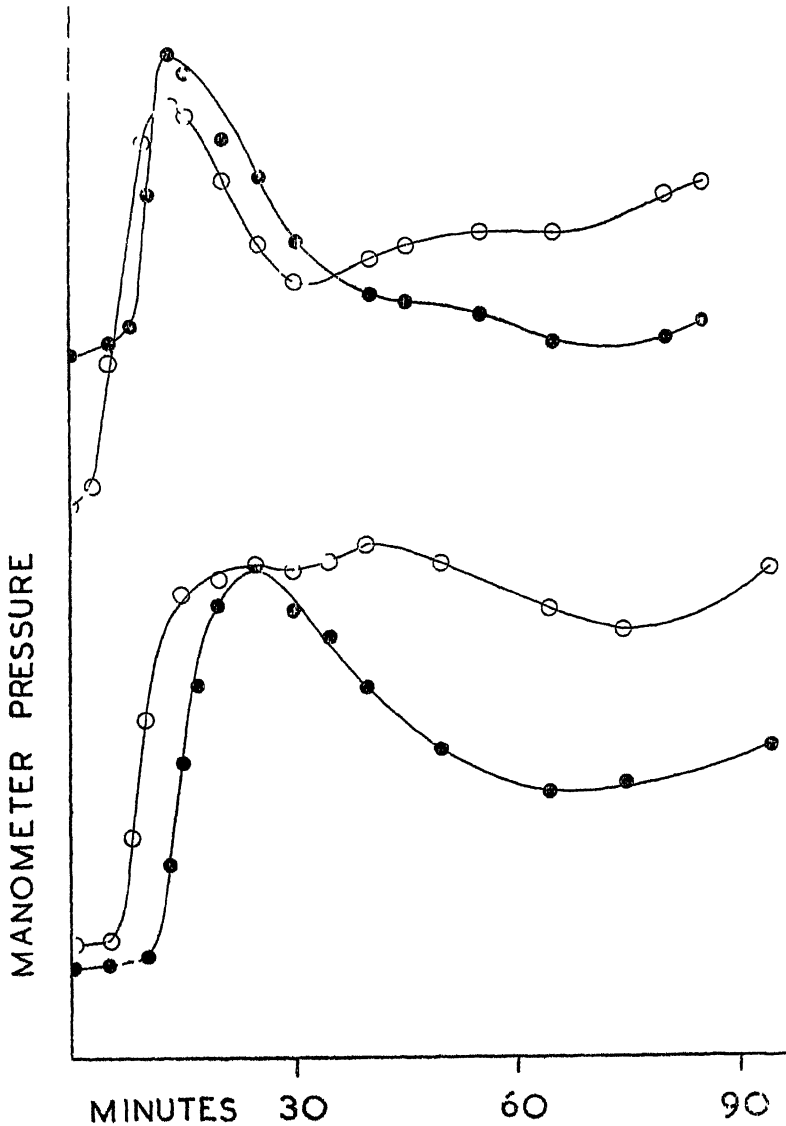


FIG. 5. Behaviour of stomata in Experiments 6 and 7.

stomata appears somewhat abnormal. We may suspect, then, that the burn inflicts slight but lasting damage on the nearer stomata.

### III. *Transmission in Cut Leaves*

Since the present work is intended to be exploratory rather than conclusive it was decided at this stage to leave the problems presented by the results just quoted, and to make a preliminary investigation of the effects on

shock-transmission of the barrier produced by a cut in the leaf. The results of a number of such experiments will be considered in the order most convenient for discussion.

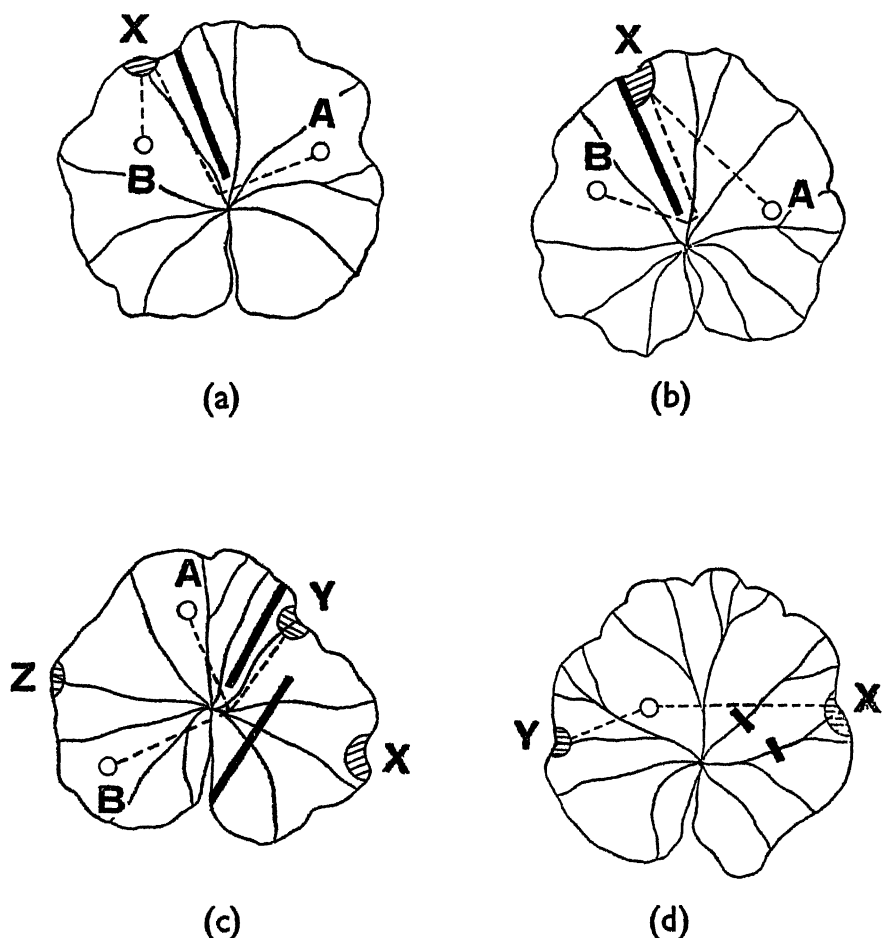


FIG. 6. Diagram of the four leaves used in Experiments 8-11.

*Experiment 8.* Consider Fig. 6 (a). The burn and porometer cups are labelled as before, and the thick black line represents a cut made *between* the main veins with a pair of scissors. The first section of Table III shows the results obtained. It will be seen that the shock has reached the farther porometer cup, and must therefore be able to pass round the cut. In general, calculations of speed of transmission will not be carried out for the experiments in this section; in this particular case the distance/threshold-time ratios for the two cups, taking the shortest possible path to the farther cup, are, as a matter of fact, equal; but this has not been found true in all experiments, and the observation is not considered to be significant.

TABLE III

Time (min.).	Expt. 8.		Expt. 9.	
	$P_2(A).$	$P_2(B).$	$P_2(A).$	$P_2(B).$
0	2.81	1.83	4.10	3.16
1	2.61	1.75	4.10	3.16
2	2.57	1.63	4.16	3.16
3	2.55	1.59	4.16	3.16
4	2.51	1.71	4.16	3.16
5	2.47	1.91	4.20	3.22
6	2.41	2.07	4.44	3.40
7	2.39	2.29	4.82	3.60
8	2.35	2.47	5.22	3.80
9	2.37	2.63	5.44	3.98
10	2.41	2.77	5.66	4.02
11	2.61	2.85	—	—
12	2.87	2.99	—	—
13	3.11	3.03	—	—
14	3.31	3.17	—	—
15	3.39	3.21	—	—
20	3.39	3.73	—	—
25	3.13	4.65	—	—

*Experiment 9.* Fig. 6 (b) and the remainder of Table III represent the results of a similar experiment; it will be seen that the shock has again passed round the barrier, though it has in this case reached the farther cup at the same time as the nearer.

*Experiment 10.* Consider the leaf shown in Fig. 6 (c). *A* and *B* represent the porometer cups, *X*, *Y*, and *Z* three successive burns. It will be observed that two cuts have been made, one of which passes *across* the main veins, whereas the other, like those in the experiments just quoted, passes *between* them. The results are given in Table IV.

TABLE IV

First burn.			Second burn.		
Time (min.).	$P_2(A).$	$P_2(B).$	Time (min.).	$P_2(A).$	$P_2(B).$
0	1.47	1.20	0	1.21	0.84
1	1.49	1.22	5	1.21	0.84
3	1.51	1.26	7	1.21	1.00
8	1.53	1.28	9	1.37	1.24
15	1.49	1.24	12	1.47	1.36
20	1.49	1.20	30	1.53	1.60
24	1.43	1.12			
30	1.41	1.04			
60	1.21	0.84			
(At this point again burnt.)			Third burn produced little effect on either cup.		

After the first burn there was a slight rise in resistance at both cups; this effect quickly passed, and the stomata continued to open. After the second burn, however, a distinct effect was observed, perhaps not great compared with that of previous experiments, but of a considerably higher order than that following the first burn, and, moreover, persistent. The first burn (*X*), then, has scarcely affected the stomata in the regions under observation, the second (*Y*)

has. This difference can hardly be due to the difference in the distances from the burn; the previous experiments have shown that the shock has no difficulty in passing round a cut between veins, and all previous experiments suggest that 10 cm. is easily covered by the shock used. We are led to suppose that the apparently insuperable barrier is the cut *across* the veins, which appears to have isolated the area which received the first burn. However, the cuts in the leaf are in this particular case extensive, and the behaviour of the leaf may have been abnormal on this account; in the experiments that follow the appropriate veins have been severed, therefore, with as little additional damage as possible.

*Experiment 11.* See Fig. 6 (*d*). The single porometer cup was attached and the leaf allowed to recover in the usual way. Cuts were then made through two of the main veins, slips of thick glazed paper being inserted through the cuts to ensure that the veins had definitely been severed. The manometer was kept under observation during this process and for 15 minutes afterwards; the stomata showed no sign of any shock, and continued to open slowly. At the end of this period the first burn (*X*) was made in the area 'isolated' by the severing of the veins; after a further 20 minutes the second burn (*Y*) was made on the undamaged side of the leaf. Table V shows the results obtained.

TABLE V

	Time (min.).	$P_2(A)$ .
First burn	0	2.57
	10	2.59
	20	2.59
	(Second burn now made.)	
Second burn	0	2.59
	2	2.61
	6	2.75
	7	2.95
	8	3.17
	9	3.37
	10	3.55

The effect is shown even more clearly than in the previous experiment; the severance of the main veins in some way isolates the area which might be expected to be served by them. Two possibilities immediately present themselves: either the area is seriously damaged by the interference with its vascular system, its stomata being possibly no longer functional, for all intents and purposes the area being a dying one; or the stomata are reacting normally within the area boundaries, the sole effect (so far as this work is concerned) being the loss of the power to transmit the heat shock out of the area. It is clearly desirable to test the reactions of the stomata *within* the area.

*Experiment 12.* See Fig. 7 (*a*). *A* is a cup within the 'isolated' area, *B* a cup out of it. The cups were attached, the leaf was left to recover, and the cuts were made, after which the stomata were kept under observation for 2 hours. The stomata in the 'isolated' area behaved in a manner precisely similar to those out of it, being completely unaffected by the cuts. Two burns were then

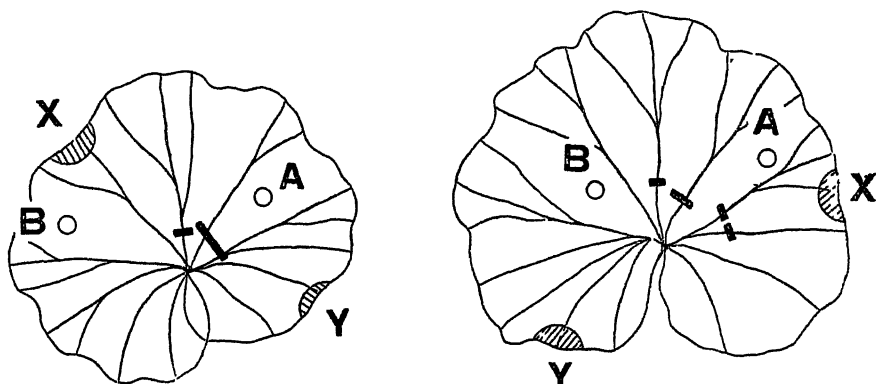


FIG. 7. Diagram of leaves used in Experiments 12 and 13.

made; the first (*X*) out of the area, the second (*Y*) in it—or nearly so. The results are given in Table VI.

TABLE VI

First burn.			Second burn.		
Time (min.).	$P_2(A)$ .	$P_2(B)$ .	Time (min.).	$P_2(A)$ .	$P_2(B)$ .
0	3.50	2.80	0	3.74	3.62
1	3.50	3.00	1	3.74	3.60
2	3.50	3.14	2	3.78	3.64
3	3.52	3.40	3	4.00	3.66
4	3.60	3.76	4	4.40	3.72
5	3.62	4.16	5	4.80	3.74
10	3.60	4.24	10	6.20	3.62
20	3.72	3.60	15	6.80	3.62
30	3.86	3.64			
60	3.74	3.62			

(Second burn now made.)

The results are striking; the burn outside the area affects strongly the stomata outside, but not those within; the burn inside the area affects—even more strongly—the stomata inside but not those without. In fact, the leaf is behaving as if it were two separate entities. It might be objected that there is in both cases a very slight effect on the other stomata, possibly representing the same shock effect diminished by distance. However, reference to experiments 2 to 5 will make it clear that the distances involved should prove no barrier to this particular type of shock, and that the shock effect at the farther porometer cup should be comparable in magnitude with that at the nearer.

*Experiment 13.* A similar experiment; the leaf is shown in Fig. 7 (b), and Table VII gives the corresponding results. It will be observed that these are precisely similar to those obtained in the preceding experiment. It is of interest to examine a graphical record of the stomata in this experiment taken over a longer period; this is shown in Fig. 8. The first curve to rise is *A*; the arrow denotes the time when the second burn, which affected *B*, was made. The two sets of stomata behave alike, each showing the now familiar oscillating movements (cf. Fig. 5).

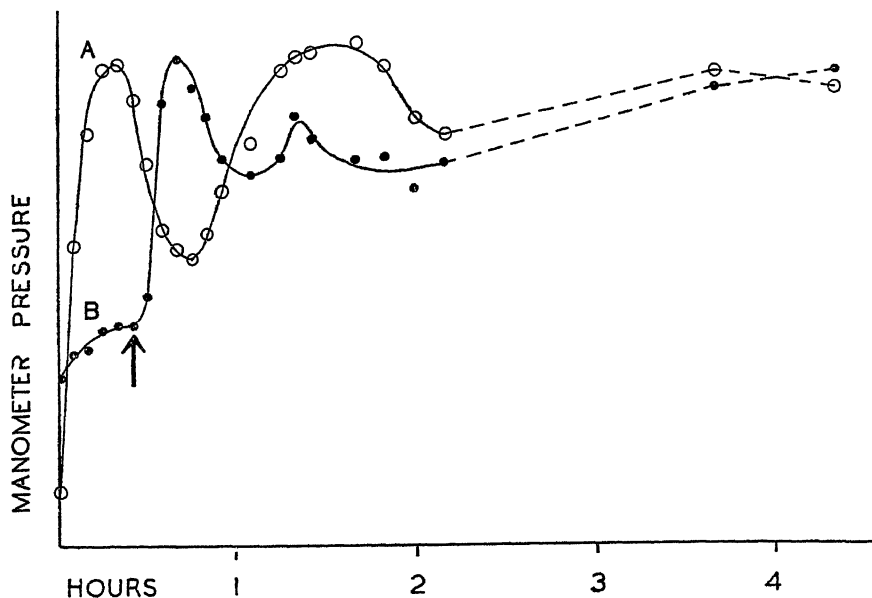


FIG. 8. Behaviour of stomata of Experiment 13 during a period of 4 hours.

TABLE VII

First burn.			Second burn.		
Time (min.).	$P_s(A)$ .	$P_s(B)$ .	Time (min.).	$P_s(A)$ .	$P_s(B)$ .
0	4.44	4.40	0	7.74	4.84
1	4.60	4.42	2	7.60	4.84
2	4.84	4.46	4	7.24	5.00
3	5.40	4.50	5	7.20	5.08
4	5.96	4.56	6	7.02	5.20
5	6.52	5.60	7	6.86	5.54
10	7.46	4.64	8	6.80	5.98
15	8.00	4.80	9	6.78	6.40
20	8.04	4.84	10	6.64	6.72
25	7.74	4.84	15	6.48	7.08
(Second burn now made.)			20	6.40	6.84

IV. *Aberrant results*

Not all results have been so convincing as the foregoing; the variability of *Pelargonium* leaves, familiar to all who have used the plant for stomatal investigations, has been well in evidence in this series of experiments. In one leaf, for example, the severance apparently had no effect at all; this phenomenon was also noted on one occasion when the burn was made immediately after the cut. In certain other leaves burning had no effect whatsoever. Further experiment will be necessary to establish the details of the phenomena associated with heat-shock transmission; but in view of the admittedly exploratory nature of the present work these aberrations will be ignored in the discussion which follows.

## DISCUSSION

The results for consideration are (1) a heat-shock produced by burning the edge of the leaf is transmitted across the leaf at a rate of the order of 0.75 cm. per minute. (2) The effect on the stomata is to cause partial closure, and this property can be used to follow the progress of the shock; with the burns used the more distant stomata were affected to substantially the same extent as the nearer; and, although the degree of closure is small compared to normal dark-closure, there is some evidence of slight lasting damage. (3) The shock passes readily round a cut in the leaf, provided that the main veins are uninjured. (4) If part of a leaf is isolated by severance of its main veins, no shock can be transmitted into or out of the 'isolated' area, although the shock reaction within the area boundaries is normal.

The relative slowness of the transmission rules out explanations based on the transmission of any form of electrical impulse; and we may postulate as a working hypothesis that some toxic substance is produced as a result of the injury to the cells, and that the transmission of the shock is due to the translocation of this toxin across the leaf. Three possible paths present themselves in a first analysis: first, by general diffusion through the cells, either of the epidermes or of the complete leaf; second, along the xylem network; third, along the phloem. The first possibility is incompatible with the speed of the process, which, though slow compared with similar phenomena in the familiar 'sensitive' plants, is nevertheless much too fast to be accounted for by simple diffusion.

The second possibility (xylem-transmission) is attractive, since there is an appreciable amount of evidence that in *Mimosa pudica* the normal ('slow') mechanism of stimulus-conduction, and in particular the mechanism of the conduction of a heat-shock due to burning, is the translocation of a wound-hormone in the transpiration stream. (Cf. the summary of the work of Ricca, Snow, and others in Stiles, 1936.) The rate of transmission in *Pelargonium* is only of the order of one-tenth of that recorded for the 'slow' conduction in *Mimosa*; but in the absence of precise information on the rate of movement of water in the xylem of *Pelargonium* the difference is inconclusive. More serious difficulties are presented by the results of the experiments with cut leaves. Since severance of main veins does not itself appear to affect the stomata in the area thus 'isolated', and since the shock-reaction within such an area is normal, it seems improbable that the area is deprived of an adequate water-supply; this point has, in fact, been investigated in another connexion by Mer (1940), who has shown that such cuts have no effect on the transpiration or water-absorption of *Pelargonium* leaves. It is clear, therefore, that the severance of the main veins cannot be said to have interrupted the xylem; if sufficient water can still be brought to the isolated area to maintain the level of transpiration, there seems no reason why the hypothetical xylem-transmitted hormone should be unable to follow the same path.

We may therefore consider the third possible path, that of the phloem.

The phloem in net-veined leaves does not, at least as an anatomically distinguishable tissue, accompany the xylem to the ultimate vascular endings; and it is therefore conceivable that a break in the main veins will represent a break in the phloem continuity. The rate of transmission is not incompatible with this theory; Dixon (1923)—in an endeavour to prove that phloem was not the path of carbohydrate translocation—calculated that in the phloem of the potato the solute particles would have to move at the rate of 50 cm. per hour. The almost exact agreement with the rate found in the experiments under discussion (45 cm. per hour) is no doubt fortuitous, and in fact other calculations by Dixon gave results varying from 20 to 140 cm./hr.; but it may reasonably be taken as evidence that the rate of transmission is of the correct order.

It is not proposed at this stage of the investigation to discuss in detail the mechanism of the effect on the stomata. The apparent absence of any decrease in magnitude of the shock at the more distant stomata can perhaps most easily be explained by an 'all-or-none' mechanism; after a small threshold-concentration of toxin is passed, the degree of closure is unaffected by the amount present. It would then be assumed that the violence of the burns was such as to enable the threshold-concentration to be attained over the entire leaf. In conclusion, therefore, we may say that the results suggest the 'heat-shock' effect in *Pelargonium* leaves to be due to a toxin, or wound-hormone, produced by the burn and transmitted to the remainder of the leaf via the phloem; but it is clear that considerable work remains to be done if the problem is to be fully elucidated.

#### SUMMARY

A heat-shock produced by burning the edge of the leaf of *Pelargonium zonale* var. *Paul Crampel* is shown to be transmitted across the leaf at a rate of the order of 0.75 cm. per minute, the progress of the shock being followed by its effect on the stomata.

The shock effect cannot be transmitted into or out of an area if the main veins serving that area have been severed, although the shock reaction within the area remains normal.

It is suggested that the shock effect is due to the production by the burnt cells of a toxin, or wound-hormone, which is translocated to the rest of the leaf via the phloem.

#### ACKNOWLEDGEMENTS

First and foremost the author wishes to thank Professor F. G. Gregory of the Imperial College of Science and Technology, at whose instigation the work was undertaken, for his stimulating criticism and advice. Part of the work was carried out at the Sir John Cass Technical Institute, and the author wishes also to thank Dr. E. de Barry Barnett for providing the necessary facilities within his department.

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# The Use of Dyes in Culture Media for Distinguishing Brown and White Wood-rotting Fungi

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With Plate I and three Figures in the Text

## INTRODUCTION

VARIOUS dyes incorporated into media have been used in laboratory practice for special purposes for a number of years. Bacteriologists were the first to recognize their use when Churchman (1912) observed that gentian violet in nutrient agar inhibited the growth of gram-positive bacteria.

A few years later Farley (1920) and Epstein and Snell (1940) used Churchman's technique to free dermatophytic fungi from bacterial contaminants, whilst McCrea (1934), Falchi (1933), and Verona (1936) tested the effect of certain dyes on the growth of this group of parasitic fungi.

The variable growth responses of fungi to dye-containing media prompted Coons (1925), Leonian (1929, 1930, 1932), Dessy (1932), and others to apply such results to the taxonomic field. In the genera *Fusarium*, *Phytophthora*, *Trichophyton*, and *Penicillium*, for example, some separation of closely allied forms has been based on their varying reactions to dye media.

The effect of the growth of the fungi on the dyes themselves has received little attention except for changes associated with drift of pH of the media. Curtin (1927), investigating the production of acids by wood-destroying fungi in culture, grew five forms—including representatives of both brown and white wood-rotting fungi—on malt-syrup agar containing a series of indicator dyes. All the fungi produced acids as shown by a change in colour of the indicators and a return to the basic colour was obtained by neutralization with sodium carbonate, except in the case of three indicators, neutral red, propyl red, and methyl red which were destroyed during growth of the fungi.

Several years after the publication of this work Refshauge and Proctor (1935), applying the results of earlier work (Coons, 1928; Leonian, 1929, 1930) to the wood-rotting fungi, attempted to establish from cultural characteristics a means of identification of a number of Basidiomycetes which had been isolated from the wood of *Eucalyptus* spp. in Victoria, Australia. One

of the media used was Czapek's agar, plus malachite green in a concentration of 0.007 per cent. They found that some of the fungi decolorized the medium during growth, and although they used this feature in the keys drawn up for identification purposes, its significance was not stressed and no further work was carried out.

The identification and naming of cultures of wood-rotting fungi isolated from samples of decayed wood without attached fruiting bodies is very difficult, particularly in Australia where little is known of the forest fungal pathogens. The detailed work of Fritz (1923) has proved invaluable in the Northern Hemisphere where identification by cultural characters is often possible; but in Australia, many typical northern fungi are absent and many endemic forms have yet to be described.

Various methods for inducing fruiting in cultures have been reported from time to time but in this laboratory success has been achieved with only a few Polyporaceae, e.g. *Fomes rudis* Berk. Abortive fructifications in the form of pores scattered over the mycelial surface on the agar are quite common among the Polypores in culture, but these atypical structures are of little value from the taxonomic viewpoint.

Cultures of *Pleurotus lampas* Berk. formed small but typical sporophores on the medium of Etter (1929). All the cultures have been unresponsive to the accelerator of Badcock (1941).

Since specific identification has often proved difficult, it was useful to separate unknown forms into the two recognized groups of wood-rotting fungi, the brown and the white rots.

Bavendamm's (1928) gallic and tannic acid media were used at first as the work of Davidson, Blaisdell, and Campbell (1938) showed that a high percentage of wood-rotting fungi could be separated into these two groups by their reaction on these media. The addition of the gallic and tannic acids to the malt agar after autoclaving (which would destroy the acids) and the use of petri dishes for the test result in a high percentage of contamination unless tedious precautions are taken.

The method set out in this paper for the separation of brown- and white-rot fungi in culture has none of these inherent disadvantages and has proved a very reliable method over the last few years. An explanation of the effect on the dyes by those fungi producing a white rot of wood is attempted.

#### EXPERIMENTAL PROCEDURE

##### *Media*

The standard medium consisted of: powdered agar, 18 gm.; malt extract (Saunders), 25 gm.; distilled water, 1,000 c.c. The required dye was added to give 0.007 per cent. concentration.

Eighteen dyes belonging to various groups, classified according to Conn, (1936) were tested: (1) Nitro dyes: picric acid. (2) Azo group: methyl orange; methyl red; orange G; Bismarck brown; Janus green; Congo red. (3) Anthraquinone group: sodium alizarin sulphonate. (4) Quinone-imine group:

methylene blue; Nile sulphate blue; brilliant cresyl blue; neutral red; thionin violet. (5) Phenyl methane dyes: malachite green, gentian violet; methyl violet. (6) Xanthene dyes: eosin; erythrosin.

Although all the dyes gave the reaction the number was later reduced to two, gentian violet and neutral red, as these gave reliable results and had none of the disadvantages characterizing some of those in the list. Phenol blue, for example, was not readily soluble in agar; methylene blue, Janus green, and thionin violet were reduced during autoclaving, and although the colour returned on cooling it was thought advisable not to use them in the standard test. The test-tubes used were similar in size, 6 in.  $\times$   $\frac{3}{4}$  in., and in each, equal quantities of the media were poured.

Inoculations were made from young vigorous cultures growing on 2.5 per cent. malt agar plates. For each fungus under test, inocula 4 in. square were placed 1 in. from the top of the slope in three tubes of each dye and three malt-agar tubes without dyes served as controls. The cultures were incubated at 25° C. for 3 weeks and notes made of their appearance at 7-day intervals.

The most suitable concentration of dye for use in the test was determined by the following experiment. Concentrations ranging from 0.001 to 0.01 per cent. at 0.001 per cent. intervals and from 0.01 to 0.07 per cent. at 0.01 per cent. intervals were prepared and tested with four fungi: *Polystictus versicolor* (W = white-rot form); *Irpex zonatus* (W); *Trametes lilacina-gilva* (B = brown-rot form); and *Polyporus anthracophilus* (B).

Neutral red agar did not restrict the growth of any of the forms in concentrations 0.001–0.03 per cent., but the effect of gentian violet was much more variable. The growth of the two brown rots and *I. zonatus* was initially restricted at a concentration of 0.002 per cent., whilst 0.008 per cent. was required before *P. versicolor* was affected.

The amount of decolorization is proportional to the amount of growth, so it follows that where growth is restricted decolorization will also be limited and therefore concentrations greater than 0.007 per cent. were undesirable. A concentration of 0.007 per cent. was finally chosen for the test as this is not too great to restrict growth over a 3 weeks' period and yet not too dilute to prevent a good colour change in that period.

#### *Method of experimentation*

Seasoned timber blocks of *Eucalyptus regnans* F.v.M. were set up according to Leutritz's (1939) soil method to test the colour of the rot produced in wood. Two blocks 2 in.  $\times$  1 in.  $\times$  1 in., with their larger surface cut across the grain and partially embedded in 400 gm. of red-brown surface loam from an *E. regnans* forest area, were contained in each screw-top jar. The soil had a moisture content of 35 per cent. before sterilization, which was carried out by autoclaving the systems at 18 lb. pressure for 30 minutes on each of three successive days.

The test fungi were grown on thick 2.5 per cent. malt agar plates. From these plates pieces of mycelium and agar were cut approximately the same

size ( $\frac{3}{4}$  in. square) and were placed at the junction of the soil and block. The jars were then incubated at 24° C. for 6 months. The jars were then opened and the blocks scraped free from adhering soil and mycelium before oven-drying for 8 days at 104° C.

*E. regnans* has a pale-coloured timber which, when autoclaved in contact with red forest loam, becomes very much darker. This is advantageous for the test as any change in colour due to the type of wood-rotting fungus present can be readily seen, and thus classification into the two groups—the brown-rot group in which the blocks were comparable in colour with the controls and the white-rot group in which they were distinctly paler (see Plate I)—made easy. In this way, unnamed fungi used in the test were separated into white- and brown-rot types and the results from the dye series were checked against these. All the species in the list given were actually tested in this way so that any error in identification might not confuse the results.

#### EXPERIMENTAL RESULTS

The fungi listed in Table I include cultures represented in the collection of the Forests' Commission of Victoria, Australia. A number of these fungi have been isolated from decayed wood of *Eucalyptus* spp., others from sporophores, and yet others have been obtained from overseas. The majority of these cultures were named, but as a check each fungus was subjected to the wood-block test. The results of the test show that the white-rot fungi during growth will decolorize dyes of any of the six types used when incorporated in a malt medium; this effect is represented by the symbol +. The brown-rot fungi, on the other hand, do not bring about any paling of the medium, an absence of effect represented by the symbol —.

#### *Constancy of results*

Nine slopes were inoculated with each fungus (three for each dye and three controls) and the amount of growth and extent of decolorization were recorded at weekly intervals.

The fungi grew readily on media containing neutral red, and if inoculated carefully (Leonian, 1932) good growth could be obtained on gentian violet media. The white-rot fungi decolorized these dyes, whilst the brown-rot fungi produced no change in colour.

The amount of decolorization for a set time varied among the white-rot forms, but three weeks was found to be essential before definite positive results were obtained for all but one of the forms tested. The exception was *Ganoderma applanatum*, and for this fungus 6 weeks' incubation was necessary before a distinctly positive result could be recorded. No brown rot caused decolorization even after 6 weeks' incubation.

Fifty-four of the fifty-six cultures tested gave results in accordance with their known grouping or, if unidentified, the result was in agreement with the wood-block test. The two fungi which did not conform to the pattern of the results were white-rot types—No. 9, an unnamed fungus, which judged

TABLE I

Dyes used: Gentian Violet and Neutral Red.

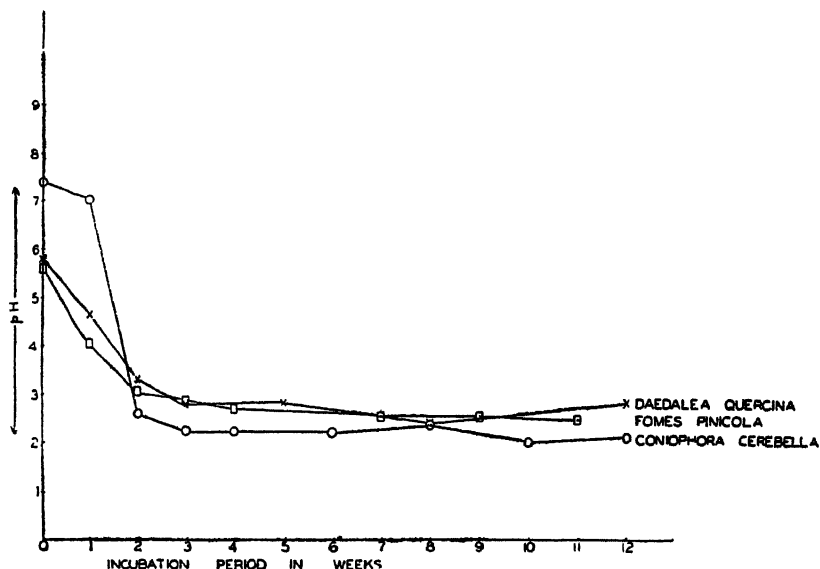
Lab. No.	Name.	Type of rot.	Dye reaction.
59	<i>Fomes ignarius</i> (L.) Fr.	White	+
69	<i>F. robustus</i> Karst.	"	+
80	<i>F. rudis</i> Berk.	"	+
60	<i>F. roseus</i> (A. & S.) Fr.	Brown	—
124	<i>F. hemitephrus</i> Berk.	"	—
82	<i>F. officinalis</i> Fr.	"	—
64	<i>Ganoderma applanatum</i> (Pers.), Pat.	White	+(6 weeks Incubation)
129	<i>Polyporus dryadeus</i> (Pers.) Fr.	"	+
71	<i>P. gilvus</i> (Schw.) Fr.	"	+
146	"	"	+
63	<i>P. hirsutus</i> (Wulf.) Fr.	"	+
34	<i>P. hispidus</i> "	"	?
143A	<i>P. melanopus</i> (Swartz.) Fr.	"	+
147	<i>P. pelles</i> Lloyd.	"	+
135	<i>P. pulcherrimus</i> Rod.	"	+
10	<i>P. anthracophilus</i> Cooke	Brown	—
33	" "	"	—
150	" "	"	—
130	<i>P. Colensoi</i> Berk.	"	—
77	<i>P. eucalyptorum</i> Fr.	"	—
125	<i>P. Hartmanni</i> Cooke.	"	—
43	<i>P. Schweinitzii</i> Fr.	"	—
26	<i>Polystictus versicolor</i> (L.) Fr.	White	+
151	" "	"	+
45	<i>Trametes cinnabarina</i> (Jacq.) Fr.	"	+
28	<i>T. lilacina-gilva</i> Berk.	Brown	—
138	" "	"	—
149	<i>T. ochroleuca</i> Berk.	"	—
24	<i>T. serialis</i> Fr.	"	—
35	<i>Daedalea quercina</i> (L.) Fr.	"	—
29	<i>Poria ferruginosa</i> (Schrud.) Fr.	White	+
67	<i>Poria</i> sp. 1.	"	+
70	<i>Poria</i> sp. 2.	"	+
68	<i>Poria</i> sp. 3.	Brown	—
27	<i>P. contigua</i> (Pers.) Fr.	"	—
132	<i>Irpex zonatus</i> Berk.	White	+
127	" "	"	+
22	<i>Stereum hirsutum</i> (Willd) Fr.	"	+
81	<i>Coniophora cerebella</i> Pers.	Brown	—
76	" "	"	—
128	<i>Armillaria mellea</i> (Vahl.) Fr.	White	+
57	<i>Collybia velutipes</i> (Curt.) Fr.	Brown	—
50	<i>Pleurotus lampas</i> Berk.	White	+
4	Unknown	"	+
6	"	"	+
9	"	"	+
16	"	"	+
18	"	"	+
47	"	"	+
49	"	"	+
74	"	"	+
13	"	Brown	—
20	"	"	—
21	"	"	—
62	"	"	—
73	"	"	—

from the wood-block test was a white-rot type, and No. 34 *P. hispidus* known to be a white-rot form. *P. hispidus* did not give a positive result on gallic and tannic acids when tested by Davidson, Blaisdell, and Campbell (1938).

## DISCUSSION OF RESULTS

1. *Decolorization by pH change*

The facts that neutral red acts as a pH indicator with a red to yellow colour change, and that it was one of the three indicators in Curtin's series which were destroyed during the growth of his test fungi, led to an investigation of the effects of pH change during growth of these fungi.



TEXT-FIG. 1. Change of pH of malt liquid inoculated with three typical brown-rot fungi. Data from Findlay et al. (1940).

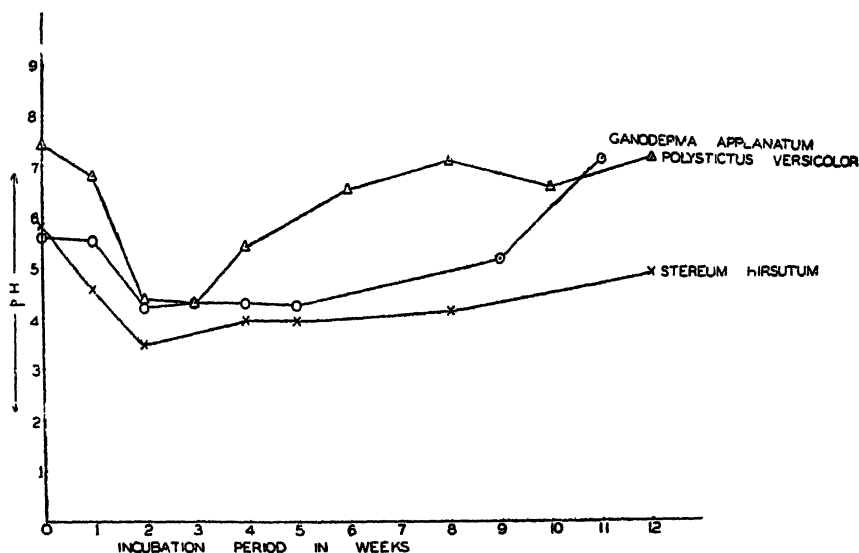
Findlay, Birkinshaw, and Webb (1940) showed that acids, e.g. citric acid, are produced during the metabolism of all wood-rotting fungi. They followed the pH change during the growth of some of these fungi and found that during the first 2–3 weeks of growth there was a drop in the pH of the medium due to the formation of these acids. This low pH persisted in the brown-rot forms (see Text-fig. 1), whereas in the white-rot forms the initial drop was followed by an increase in pH to the region of neutrality after 12 weeks' incubation; this it was suggested was due to the destruction of the acids by oxidases (see Text-fig. 2).

These facts must be considered in relation to the experiment described above. Of the two dyes chosen, gentian violet is a 'poorly defined mixture of violet rosanilins' (Conn, 1936) which does not change in colour over a pH range of 3–6. Above pH 6 it merely becomes more reddish in colour. Neutral red, on the other hand, is a weakly basic dye which acts as an indicator with a pH range of 6.8–8.0, being red in acid and yellow in alkali.

If acids resulting from fungal metabolism brought about a change in colour of the dyes, all the fungi should produce such a change (see Text-figs. 1 and 2),

but no decolorization was recorded for the brown-rot forms. Moreover, it takes 12 weeks for the media supporting the growth of the white-rot fungi to reach pH 7 and decolorization was apparent from three weeks onwards.

As already stated, Curtin (1927), by incorporating indicator dyes in their basic colour state with malt-syrup agar, showed that acids were produced by



TEXT-FIG. 2. Change of pH of malt liquid inoculated with three typical white-rot fungi. Data from Findlay et al. (1940).

wood-rotting fungi during growth. A change in colour of these dyes during growth of the inoculated fungi indicated the production of acids if the basic colour could be regained on addition of sodium carbonate.

As it was not possible to reproduce Curtin's experiment because insufficient details of the method were given (e.g. standard pH of the medium, concentration of indicator, &c.) an alternative experiment was planned along closely similar lines. For this experiment the malt-agar medium was used and the indicators—methyl orange, Congo red, methyl red, sodium alizarin sulphate, litmus, rosolic acid, and neutral red—were added in 0.004 per cent. concentration.

Four test fungi were used: *P. versicolor* (W); *S. hirsutum* (W); *P. anthracophilus* (B); and *T. lilacina-gilva* (B). The inoculated tubes were incubated at 24° C. for 3 weeks, after which their appearance was noted. The agar in the tubes was then melted, the mycelium removed, and 2 c.c. of 5 per cent. sodium carbonate added to each tube. Uninoculated control tubes were treated in the same way to serve as standards.

The results obtained are set out in Table II. The colours given are those of Ridgway's Colour Standards and Nomenclature.

The malt agar had a pH of 5.4 with the result that the first five of the seven

TABLE II

Indicator.	pH range.	Acid-alkali change.	Colour in malt agar.
Methyl orange . . . .	3·1-4·4	Red-yellow	Honey yellow
Congo red . . . . .	3·0-5·0	Bluish-red	Rufous colour
Methyl red . . . . .	4·2-6·3	Red-yellow	Cinnamon buff
Sodium alizarin sulphonate	3·7-5·2	Yellow-violet	Vinaceous-drab
Litmus . . . . .	5·0-8·0	Red-blue	Light pinkish cinnamon
Rosolic acid . . . . .	6·9-8·0	Brown-red	Honey yellow
Neutral red . . . . .	6·8-8·0	Red-yellow	Acajou red

Malt agar without indicator = chamois

Indicator.	Colour of reverse after incubation.		Results of Na <sub>2</sub> CO <sub>3</sub> test.	
	Brown rot.	White rot.	Brown rot.	White rot.
Methyl orange . . . .	Unchanged	Unchanged	As control	Paler than control
Congo red . . . . .	Unchanged	1 in. decolorized	„	No return of colour
Methyl red . . . . .	Unchanged	Unchanged	„	„
Sodium alizarin sulphonate	2 in. decolorized	Completely decolorized	„	No return of colour
Litmus . . . . .	Unchanged	2 in. decolorized	„	„
Rosolic acid . . . . .	Unchanged	Unchanged	„	„
Neutral red . . . . .	Unchanged	1 in. decolorized	„	„

indicators were in their basic state and the last two in their acidic. Therefore at this low concentration four of the indicators—methyl orange, methyl red, litmus, and rosolic acid—brought about very little change in the colour of the malt agar (chamois colour) as shown in column 4.

Sodium alizarin sulphonate was the only indicator in its basic colour which was sensitive to the acids produced by the brown-rot fungi (column 5). The white-rot fungi brought about decolorization of the four brightly coloured indicators, and when the sodium carbonate test was applied to the decolorized portion of the agar from these tubes no colour comparable to the standards was obtained, showing that the indicators had been completely destroyed.

Every indicator when tested with sodium carbonate after growth of the brown-rot fungi gave a colour similar to the control standards including the sodium alizarin sulphonate. Therefore, in contrast to the effect of the white-rot fungi, pH changes had taken place only where the indicators were affected.

These results are not in agreement with those of Curtin (1927), who found that the brown-rot fungi and the white-rot fungi alike brought about the destruction of methyl red and neutral red, whilst the colour of the sodium alizarin sulphonate responded to the sodium carbonate test after growth of the white-rot fungi. His results could not be reproduced in this laboratory perhaps because different fungi were used in the test. We were not able to obtain his species for our experiment.

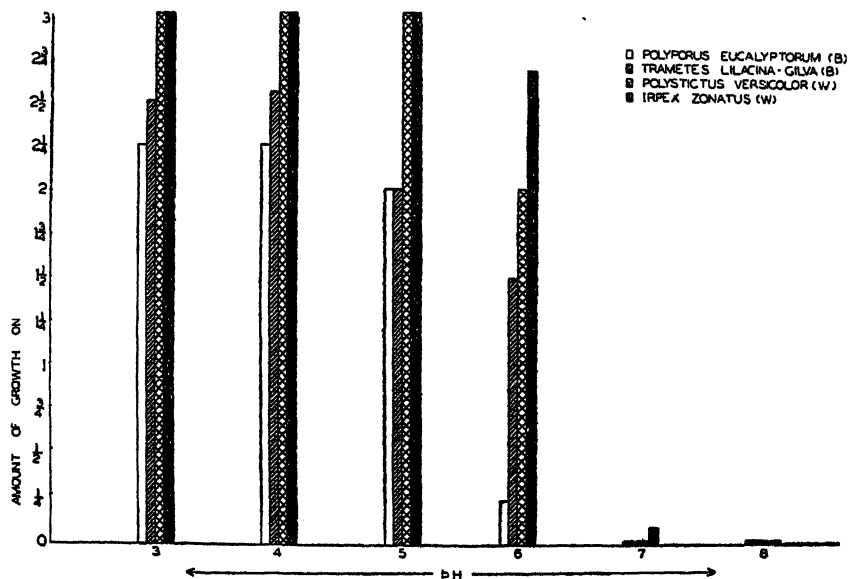
The effect of the pH of the medium on the growth of these fungi in relation to dyes was determined by a further experiment in which the media containing dyes in 0·001 per cent. concentration was adjusted over a pH range of 3-8.<sup>1</sup>

Four fungi—*P. versicolor* (W), *I. zonatus* (W), *T. lilacina-gilva* (B), and

<sup>1</sup> The latter part of this work was carried out in collaboration with Miss K. Law of the Biochemical Department of Melbourne University.

*P. eucalyptorum* (B)—were selected for inoculation. Three slopes of each dye at each pH, with a comparable set of plain malt agar controls, were inoculated for every fungus, and incubated at 24° C. for 3 weeks. It was found that the best growth of the fungi took place from pH 3 to pH 5. At pH 6 limited growth was recorded, but none occurred at pH 7 and 8 (see Text-fig. 3).

The brown-rot fungi did not bring about loss of colour of the dyes at any



TEXT-FIG. 3. Fungal growth at varying pH levels.

pH, but, where growth was recorded for the white-rot fungi, decolorization took place irrespective of pH.

This experiment shows that, although the pH of the medium influences the amount of mycelium produced by these fungi, the white-rot fungi will decolorize the dye to some extent whenever growth takes place.

## 2. Decolorisation by oxidation-reduction

The possibility existed that the decolorization of some at least of the dyes was due to reduction, as in the well-known Thunberg experiment with methylene blue. It might be, for example, that under the conditions of the experiment, anaerobic conditions were created in the medium. However, experiment shows that the decolorization is not reversible by oxygen and it appears to be one of complete breakdown of the dye molecule. This being so, consideration of the dye redox potentials would be of little value. Experiment has, however, shown that the following dyes, chosen for their position on the redox scale, are completely decolorized by the white-rot fungi: neutral red ( $-0.325$ ); Janus green ( $-0.255$ ); methylene blue ( $+0.011$ ); brilliant cresyl blue ( $+0.047$ ); thionin blue ( $+0.063$ ); phenol blue ( $+0.224$ ).

### 3. Decolorization by enzymic action

There remains the possibility that the dyes are oxidized by the enzyme systems of the white rots. Some further experiments support this view. It was impossible to grow the test fungi—*P. versicolor*, *S. hirsutum*, *P. anthracophilus*, and *T. lilacina-gilva*—in an atmosphere of pure hydrogen (MacIntosh and Filde's jar) or in pure carbon dioxide, but all four of the above fungi grew on agar slopes contained in a desiccator filled with commercial nitrogen. The desiccators after 3 weeks' incubation contained about 1 per cent. oxygen and 3.5 per cent. carbon dioxide. Growth was comparable with that of the controls in air, but there was no decolorization of the two dyes with either the white or brown rots. When removed from the desiccator the tubes containing the white rots showed, over the subsequent 24 hours, an amount of decolorization which normally took 5 days to produce.

This experiment was repeated with different partial pressures of oxygen. The results showed that the decolorization of the dye and the rate of fungal growth were unaffected by a change of oxygen concentration from 6 to 100 per cent. Between 1–4 per cent. of oxygen, however, although growth was not markedly affected, no decolorization took place with the white-rot fungi, but a small amount resulted from concentrations 4–6 per cent. Complete absence of oxygen was accompanied by cessation of growth.

It seems clear that the white rots have a greater oxidizing power than the brown rots. It seems probable that the oxidation is carried out after the production of an extracellular enzyme system rather than in the living cell. The experiment just described also suggests that the enzyme system is produced by the fungus growing in very low oxygen concentrations, but only acts on the dyes when the oxygen concentration is raised above 4–6 per cent. Further work on the nature of this oxidase system is proceeding.

## DISCUSSION

It has been assumed by the majority of workers dealing with timber decay that the fundamental difference between the white and the brown wood-destroying fungi lies in the possession of an active oxidase system by the white-rot forms. The evidence for this is often indirect. Thus Findlay et al. (1940) explain the rise in pH of the medium, known to occur during the growth of *white-rot* fungi, as due to the oxidation of preformed organic acids (Text-figs. 2 and 3 are plotted from their data). Our work provides more direct evidence in that the white rots are shown to be capable of destroying added dyes, probably by an oxidation reaction.

Boswell (1941) put all the wood-rotting fungi into one large group, with the white-rot fungi at one extreme and the brown-rot fungi at the other. He suggests that although both types possess oxidase and hydrolytic enzymes they differ in the relative activities of these enzymes.

Detailed chemical analyses on the effect of decay on wood (Campbell, 1932) show that the brown-rot fungi form a homogeneous group in which the type

of attack on timber is comparable to weak acid hydrolysis. The white-rot fungi, on the other hand, can be subdivided into three classes which are dependent on the sequence of attack on the wood components. In the first group the lignin is attacked in the initial stages of decay, e.g. *Polystictus versicolor*; in the second the cellulose is broken down first, e.g. *Armillaria mellea*, and in the third group, attack on lignin and cellulose is simultaneous, e.g. *Polyporus hispidus* and *Ganoderma applanatum*.

The decolorizing of dyes by white-rot fungi seems to be characteristic of the forms placed in his groups 1 and 2, and it is interesting to note that the two fungi he cites as typical of his third grouping are *Polyporus hispidus* and *Ganoderma applanatum*. Cultures of strains of both of these fungi were included in the fungi tested and they were the only exceptions to the general results.

*Ganoderma applanatum* required 6 weeks' incubation before any definite decolorization could be recorded and *Polyporus hispidus* unlike any other white-rot type tested never gave a positive reaction to the dye.

The dye test described in this paper therefore tends to support Campbell's classification of timber-rotting fungi, while providing a much more rapid and convenient method of identifying the group to which they belong.

#### SUMMARY

A method has been developed for distinguishing in culture between white and brown wood-destroying fungi.

A malt agar medium with 0.007 per cent. concentration of various dyes incorporated with it was decolorized by white-rot fungi whilst the brown-rot fungi did not bring about any loss of colour. Gentian violet and neutral red were the most satisfactory of the dyes tried. The colour of the rot produced by the test cultures was determined by the wood-block test.

The complete decolorization of the dye takes place only when the oxygen concentration above the medium was greater than 6 per cent. The decolorization is probably due to the production, by the white-rot fungi, of an extra-cellular oxidase system. This system can be produced by fungi growing at oxygen concentrations below 6 per cent.

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## EXPLANATION OF PLATE I

Illustrating the article by A. Preston and E. I. McLennan on The Use of Dyes in Culture Media for distinguishing Brown and White Wood-rotting Fungi.

Fig. 1. Seasoned wood blocks of *Eucalyptus regnans* before autoclaving.

Fig. 2. Control blocks after six months' incubation, showing darkening during autoclaving.

Fig. 3. Blocks attacked by white-rot fungi.

Fig. 4. Blocks attacked by brown-rot fungi.



PRESTON AND McLENNAN—WOOD-ROTTING FUNGI



## Studies in the Development of the Inflorescence

### IV. The Capitula of *Hieracium boreale* Fries and *Dahlia gracilis* Ortg.<sup>1</sup>

BY

W. R. PHILIPSON

With Plate II and four Figures in the Text

*Hieracium boreale* Fries

*HIERACIUM boreale* Fries was selected for study not only as an example of the Liguliflorae but also as a member of the Compositae with an elaborately branched inflorescence borne on an elongated leafy stem. The plant perennates as a rhizome, the terminal bud of which develops in spring into a tall, unbranched, leafy shoot. A lateral bud at the base of this shoot continues the rhizome, which is therefore a sympodium. The leaves gradually decrease in size towards the top of the main axis, which ends in a single capitulum. The axillary buds of several of the upper leaves develop into branches which also end in capitula. Tertiary flowering branches are commonly developed.

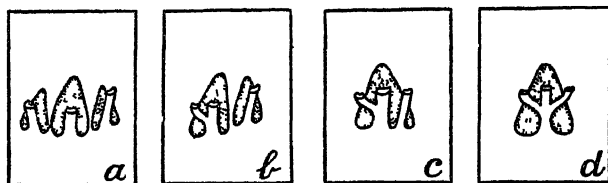
#### (a) *The vascular supply of the bracts*

It is not proposed to describe the development of the individual capitula in this species, for it does not differ fundamentally from that of *Bellis* (Philipson, 1946). Only one feature calls for special mention, namely, the development of the vascular supply of the involucre. The upper leaves (bracts) of the main axis which subtend lateral flowering branches continue the  $5/8$  phyllotaxis of the lower leaves. This phyllotaxis is continued, at least approximately, above the last lateral branch by about twelve small bracts which resemble the involucre bracts in appearance and in lacking meristematic tissue in their axils. Each involucre bract receives a single trace from the cylindrical stele of the receptacle. This trace divides within the blade of the bract to form lateral veins (Text-fig. 1d). It is interesting to follow the transition to this state from that of the vegetative leaves and the bracts which subtend flowering branches, in which three traces leave the stele independently, so that three distinct leaf-gaps are formed at their insertion (Text-fig. 1a). In one series studied the transition had already begun in the first budless bract. The anodic lateral trace arose from the stele with an independent gap, whereas the cathodic lateral trace arose as a branch of the mid-trace (Text-fig. 1b). In the second budless bract the anodic lateral trace still arose independently from the stele, but now from the same gap as the mid-trace (Text-fig. 1c). In the third budless bract, and in all higher bracts, including the involucre bracts, the vascular supply was as shown in Text-fig. 1d.

The vascular supply of each involucre bract is independent of its neighbours,

<sup>1</sup> Part of a thesis approved for the degree of Ph.D. in the University of London, 1947.

there being no linkage between the lateral traces of adjacent involucre bracts as in *Succisa* (Philipson, 1947), nor between their mid-traces as in *Bellis* (Philipson, 1946).



TEXT-FIG. 1. *Hieracium boreale* Fr. a-d, diagrams of the vascular bundles of bracts inserted successively higher on the peduncle, (a) being the highest bract subtending a flowering branch.

As in *Bellis*, the vascular supply to each flower primordium consists of a single trace, each leaving a single gap in the stele of the receptacle.

(b) *The origin and development of the inflorescence branches*

The principal feature of interest in this species is the formation of the branches which make up the terminal compound inflorescence, a feature absent from *Bellis*. These branches develop from the meristems which occur in the axils of the leaves and bracts, except those immediately preceding the involucre. These meristems, like the leaf primordia which subtend them, are formed acropetally, but they remain undeveloped and dormant until the apex of the main axis becomes visibly committed to flowering. Pl. II, Fig. 1 represents a longitudinal section through the apex of a stem which is giving rise to leaf primordia. This stem apex was gathered on May 26, 1943, from a well-developed stem, which, judging from the observed development of precisely similar stems, would have been plainly committed to the formation of a capitulum in the course of a week or so. On the left of the section a leaf rudiment ( $l_2$ ) is cut medianly; it is well developed, yet the meristem in its axil has not swollen into the primordium of an axillary bud, but remains as an arc of meristematic cells submerged in the tissue of the axis ( $b_2$ ). It is interesting to note that this arc of meristematic tissue clearly originates as a residue of the apical meristem (see Pl. II, Fig. 5, which shows a portion of the same apex at a higher magnification). The younger rudiment at  $l_1$  also subtends an arc of narrow cells ( $b_1$ ) which are continuous with, and form the flank of, the apical meristem. Immediately above, two adjacent periclinal divisions can be seen at  $p$  in the second tunica layer (Pl. II, Fig. 5); these represent the first stage in the production of a leaf primordium by the apical meristem. The cells in the 'axil' of this incipient primordium are not visibly differentiated from other cells of the meristem which is giving rise to the new leaf.

Pl. II, Fig. 2, represents the apex of a plant gathered 9 days later (June 4, 1943). An examination of the series of sections from which this was selected shows the apex to be surrounded by the primordia of the involucre bracts and therefore to have been committed to reproduction. This section was selected for illustration because it passes medianly through two leaf rudiments. The bud in the axil of the upper of these ( $b_1$ ) is still undeveloped, but that of

the older leaf has begun to enlarge ( $b_2$ ). In the section shown in Pl. II, Fig. 3, gathered 9 days later in the same season (June 13, 1943), the bud in the axil of an upper leaf ( $b_1$ ) can be seen to have developed, and, in fact, to have become larger than a bud some distance below it ( $b_2$ ). Although it is larger it is not so advanced in development since prophyll primordia are present only in the lower bud. Finally, Pl. II, Fig. 4 (gathered June 24, 1943), shows that the uppermost buds have outstripped the older buds below them in development as well as stature, and observations on mature plants confirm that the capitula terminating the lateral branches come to flower in a basipetal succession. The flowering branches become successively less strongly developed downwards, those in the axils of a few of the lower bracts (or upper leaves) often remaining in a semi-expanded condition, but the majority of the foliage leaves subtend meristems which never develop.

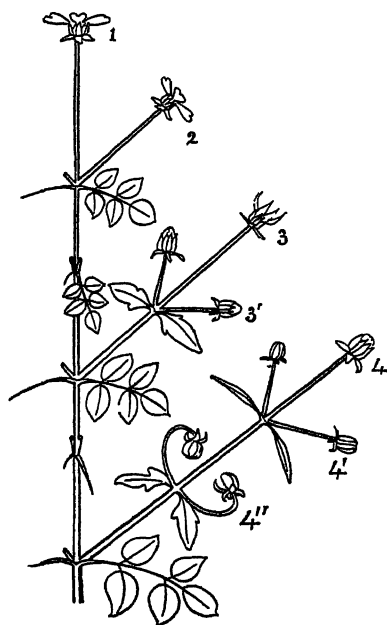
It has already been shown that the buds from which these flowering branches spring originate as residues of the apical meristem. As the stem increases in length the cells of the cortex and pith mature and become increasingly vacuolated, leaving between them the less mature cells of the provascular meristem (Pl. II, Fig. 5). The meristematic cells of each leaf primordium similarly become affected by maturation, an extension of the vacuolation of the cortex marking the dorsal mesophyll ( $d$ ), and the ventral mesophyll ( $v$ ) being formed by an extension of the vacuolation of the pith. This extension 'pierces' the provascular meristem to form the gap corresponding to the leaf primordium ( $g_1$ ). The provascular meristem on either side of the gap remains continuous with the apical meristem. At this stage the bud meristem is still continuous with the apical meristem ( $b_1$ ): maturation of the cortex now isolates the bud from the apex ( $b_2$ ) and a second extension of the pith unites with this cortex to form the 'bud-gap'. In tangential view the bud meristem now appears as an elongated band of deeply staining tissue. An examination of series of sections shows that the two ends of this band remain continuous with the provascular meristem of the main axis; that is to say, the bud meristem stands between the leaf-gap and the bud-gap like the rung of a ladder. When the bud primordia begin to enlarge they remain entirely meristematic, and retain their connexion with the sides of the gap. As the dome of the bud enlarges into a cylindrical bud primordium, cell-maturation encroaches into its central tissues from the pith of the main axis, but the two lateral connexions remain to form the mid-traces of the two prophylls.

#### *Dahlia gracilis* Ortg.

*Dahlia gracilis* was selected for study as a second example of the non-ligulate Compositae because its decussate phyllotaxis results in its capitula being arranged similarly to those of *Succisa* and *Dipsacus*, which it also resembles in possessing well-developed bracts below each floret. It is therefore possible to compare the development of the inflorescence in the Dipsacaceae more closely with *Dahlia* than was possible with *Bellis* (Philipson, 1946, 1947). It should be borne in mind, however, that the life-forms of the plants being

compared are dissimilar. *Succisa pratensis* and *Dipsacus fullonum* are rosette plants, no doubt through the action of light in inhibiting the elongation of their internodes (see Greulach, 1942), but whereas the reaction of the main

axis of *Dipsacus* changes, possibly as in beet by the action of low temperature, so that it comes to elongate as the aerial flowering shoot, that of *Succisa* remains permanently in the rosette stage, the lateral branches alone elongating as flowering shoots. In *Dahlia* the internodes of the plumular axis elongate as they are formed and the main axis of the plant comes to flower in its first season, the plant persisting by means of basal buds in connexion with the root-tubers.



TEXT-FIG. 2. *Dahlia gracilis* Ortg. Diagram indicating the sequence of flowering. 1-4, the capitula terminating the primary axis and the secondary axes. 3', 4', 4'', capitula terminating tertiary axes.

plant. The behaviour of the lateral buds is different from that of *Hieracium*, for they do not remain dormant until the apex of the main axis is committed to flowering, but form prophyll and leaf primordia. The habit of the plant is therefore to form a pyramidal leafy shoot. When these leaves and branches have been laid down, but before most of them have expanded and while the plant is still small, leaf-formation ceases at the apex of the main shoot and the primordia of the involucre bracts are laid down. The apices of the secondary branches then become committed to capitulum-production in downward succession, and similar gradients can be traced down the tertiary branches of each secondary branch if any are present. As a result, the apex of every branch on the aerial shoot becomes committed to flowering within a very short time of the first sign of capitulum-formation on the main axis. Text-fig. 2 indicates this mode of development.

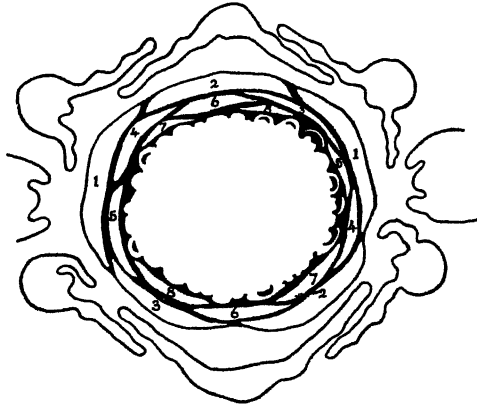
#### (b) The formation of the involucre

As is well known, the involucre of *Dahlia* consists of an outer reflexed whorl of bracts and an inner erect series (Pl. II, Fig. 6). In *Dahlia gracilis* the outer

#### (a) The formation of secondary and tertiary branches

The plants studied were grown from seed, and all sections used in studying the development of the capitulum were taken from the capitulum which terminates the plumular axis in its first year of growth. The secondary, and even the tertiary, branches also become transformed into capitula and their apices were studied in connexion with the branching of the flowering part of the

series consists of five members and the inner of eleven. The innermost two or three involucre bracts are very small and resemble the receptacular bracts which subtend the flowers. The involucre bracts are arranged in pairs, which continue at first the decussate phyllotaxis of the foliage leaves. Text-fig. 3 is from a camera-lucida drawing of a section through the uppermost pair of leaves and a young capitulum. The bracts are numbered in the order of their insertion, which is presumed to correspond with the order of their initiation.



TEXT-FIG. 3. *Dahlia gracilis* Ortg. Camera-lucida drawing of a section passing through two pairs of leaves, the sixteen involucre bracts, and the base of the receptacle with receptacular bracts and flower primordia. The pairs of involucre bracts are numbered in the order of their insertion on the receptacle.

Examination of younger capitula, in which the full complement of bracts had not been developed, confirms this order of initiation. No buds appear in the axils of the involucre bracts and no residual meristematic cells persist there. It is interesting that one of the uppermost pair of leaves often lacks a flowering branch in its axil. Pl. II, Fig. 7 represents a transverse section through the uppermost pair of leaves in the axils of each of which buds were laid down, but of which one has become aborted. One of the lateral buds in Pl. II, Fig. 8 can also be seen to be aborted. These aborted buds are frequently carried up on the pedicel above the leaf-axil.

The vascular supply of the foliage leaves is similar to that of *Succisa*, that is to say, the three traces of each leaf are inserted into the stele with a separate gap, and the two bud-traces are inserted on the margins of the gap corresponding to the midrib. Each involucre bract also receives three separate traces from the stele of the pedicel. The lateral traces of adjacent bracts are connected by transverse strands.

#### (c) *The formation of receptacular bracts and florets*

The most interesting feature in the development of this type is the initiation of the receptacular bracts and the floral primordia in their axils. The similarity of these structures (Pl. II, Fig. 6) with those found in *Dipsacus* allows of their direct comparison.

The growth of the receptacle is similar to that of all capitula studied: that is to say, the uniform meristematic mantle contributes to the growth of the parenchymatous core, and moves outwards as a meristem of more or less constant depth (Pl. II, Fig. 8). It is in this peripheral meristem that the bract and floret primordia arise. The primordium of a bract is first visible as a slight swelling caused by divisions in the inner of the two tunica layers (Pl. II, Fig. 9). These divisions are periclinal as well as anticlinal, so that the bract primordium comes to be enclosed in a single tunica layer. Immediately above the swelling the two tunica layers can be seen to have divided anticlinally several times forming a plate of narrow cells (*f*). These cells represent the earliest indication of the floret primordium. Periclinal divisions are absent so that both layers of the tunica are preserved. Pl. II, Fig. 10 represents a bract and its floret at a later stage of development. The bract is no longer wholly meristematic, for the dorsal and ventral mesophyll has become vacuolated, leaving a central provascular meristem. The floret primordium has been isolated from the meristem of the bract by this vacuolation and can be seen to have begun to grow outwards as a visible primordium. A comparison of this description and the photographs with those previously published for *Dipsacus* (Philipson, 1947) will show that the development of the receptacular bracts and florets is identical in *Dipsacus* and *Dahlia*.

#### DISCUSSION

With the examination of these further two types taken from the Compositae it is possible to extend the comparison of the development of the capitulum in this family with that in the Dipsacaceae which was made in an earlier paper in this series (Philipson, 1947). These types further emphasize the general similarity in the course of development in the two families. In particular, the growth of the receptacle and the origin of the vascular strands of the bracts and florets are the same, and in addition, the development of the receptacular bracts in *Dahlia* and their relation to the floret primordia is identical with those in *Dipsacus*. On the other hand, one of the principal differences between the capitula in the two groups, namely, the absence of buds in the axils of the involucre bracts, is confirmed by both *Hieracium* and *Dahlia*. It is particularly interesting that a Composite in which each floret is subtended by a well-developed receptacular bract should lack meristems in the axils of the involucre. For a brief period in the growth of the axis no part of the apical meristem becomes detached as the primordia of lateral buds. Both before, when foliage is being formed, and after, when the receptacular scales are appearing, axillary buds are formed. A tendency for 'sterility' of the axils of the bracts to extend below the involucre was seen in *Bellis*, where the two uppermost foliage leaves lacked buds. A similar trend is seen in *Dahlia*, in which one of the uppermost pair of branches is frequently aborted. In *Hieracium* this feature is more marked, for several sterile bracts separate the uppermost flowering branch from the involucre. McLean Thompson (unpublished MS.) refers to the presence or absence of meristems in the axils of

the bracts of *Forsythia*, and suggests how different forms of inflorescence may be brought about by the presence or absence of axillary meristems and their development at different rates.

An interesting, and unexpected, feature of the capitula of the three Compositae investigated is the considerable variation in the vascular system of the involucre bracts. In *Hieracium* the supply to each bract is independent of its neighbours and all the veins originate from a single trace: in *Dahlia* the bracts are supplied by three traces, the lateral of which make connexions with those of adjacent bracts: in *Bellis* each bract also receives three traces, and although the veins to each bract make no junction with their neighbours, a large vascular strand encircles the top of the peduncle, and unites the traces of all the involucre bracts. A systematic examination of the Compositae from this point of view would show whether these variations are of taxonomic value or whether they reflect the morphological type of the involucre, as, for example, whether free or united, broadly or narrowly inserted, &c., differences to be found within each tribe of the Compositae.

Sinnott (1914) has shown that the number of traces to the foliage leaves (whether one, three, or more) is a character of considerable value in defining large plant groups. Both the Compositae and the Dipsacaceae are characterized by possessing three traces to their leaves, whereas the Rubiaceae and the Campanulaceae have the less common single leaf-trace. Sinnott records a number of groups in which the number of traces is variable. The present study of *Hieracium* reveals an example of a plant in which the number of traces is related to the size of the leaf, for as the bracts become smaller the number of traces decreases first to two, and finally to one.

*Hieracium boreale* shows very clearly the two gradients which were discussed when the branching of *Valeriana officinalis* was described in this series of papers (Philipson, 1947a). The size of the foliage leaves falls off steadily towards the top of the stem, while the degree of development of the mature lateral flowering branches falls off in the reverse direction. The axillary buds on the main axis are under very strict control, by means of which the habit and life-form of the plant is regulated. The buds in the axils of the foliage leaves are dormant and remain so under normal circumstances, except for one at the extreme base of the plant which becomes enlarged after flowering has been achieved and forms an extension of the sympodial rhizome. The behaviour of this bud was not relevant to the present inquiry and was not studied in detail. The buds in the axils of the higher leaves were studied and their behaviour has been described. When the apex of the main axis is committed to reproduction the buds in the axils of the higher leaf primordia are no longer inhibited but enlarge as they are laid down in acropetal succession. Although the buds are initiated and begin their development in acropetal succession, their further development goes on at rates which decrease in a basipetal direction. As a result the uppermost buds quickly overtake and pass those below them, and the mature capitula which terminate the side branches come to flower in a basipetal sequence.

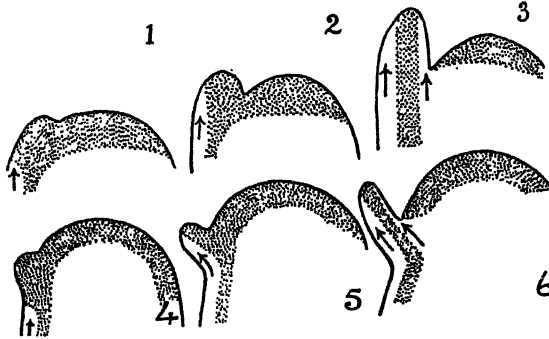
The control of the buds in *Dahlia gracilis* is completely different from that just described for *Hieracium*, so that the mature plants are of very different habit. In *Dahlia* the plumular axis (and no doubt the shoots produced from the buds on the root-tubers, though these were not studied) develops into an aerial leafy stem. Once the plant has grown beyond the seedling stage the axillary buds do not remain dormant, but develop prophylls and leaf primordia shortly after they are formed below the stem apex. As these buds grow out into leafy shoots the habit of the plant is that of a pyramid of ever-increasing size. When the plant enters the reproductive phase it is interesting that all active apices on the plant are committed to capitulum-production, not simultaneously, but in basipetal sequence down the secondary branches, and in similar sequences down the tertiary branches.

The contrast between the behaviour of the lateral buds in these two Composites suggests that a study of the inhibition and release of buds might be of considerable interest in connexion with the development of life-forms. The buds of *Dahlia* appear to be under little restraint, each growing as it is formed and reaching the flowering condition in accordance with a basipetal gradient. Many annual plants have been examined and appear to conform to this habit: the plants of *Dahlia* would be annuals if a basal bud was not reserved in connexion with the root-tubers. It is in the possession of these buds that *Dahlia* indicates the possibility of the differential development of buds which would result in their reservation and orderly release, as seen in diverse life-forms, culminating in the complex co-ordination of buds seen in forest trees, in which, for example, buds may remain dormant or develop into long or short shoots bearing foliage or flowers.

In describing the origin of the provascular strands of the involuclral bracts of *Bellis* (Philipson, 1946) by the cleavage of a peripheral meristem into peripheral and provascular portions, this mode of development was contrasted with that to be found in foliage leaves. Subsequent work has confirmed that mode of development, but acquaintance with a wider range of forms, in which bracts and foliage are not so distinct as in *Bellis*, has led to the view that the origin of the provascular strands is essentially similar in leaves and bracts, differences in appearance being due to the relative size of the primordia and the apex and to the distance from the apex at which the primordia originate. In the vegetative apex the progress of cell maturation is crowded close to the apex where a number of primordia are present so that it is difficult to make out the origin of the first provascular strands. Text-fig. 4 indicates the essential similarity between the origin of the provascular meristem in a leaf and a bract. The similarity of these diagrams of foliage-leaf primordia to the photograph of the apex of *Hieracium* (Pl. II, Fig. 5) is evident, although they were based on the development of the leaf in several species rather than in *Hieracium* in particular.

Several problems connected with the origin and early development of axillary buds remain without final solution, in spite of several recent investigations. Firstly, there is conflicting evidence as to the origin of the meristematic cells from which the buds arise. Wardlaw working on ferns (1943 and sub-

sequent papers) has described the origin of buds from meristems 'detached' from the apical meristem. In the present investigations the origin of flowering-branch buds has always been of this type. On the other hand, flowering-branch buds have been found to develop much earlier than vegetative buds (see



TEXT-FIG. 4. Diagram indicating the similarity between the origin of a foliage leaf (components 1-3) and of a bract (components 4-6). Arrows indicate the direction in which the maturation of the cells is progressing. See text for further explanation.

particularly *Valeriana* (Philipson, 1947*a*)) and might therefore be expected to show a closer relation to the apical meristem. Nevertheless, the meristems in the axils of the foliage leaves of *Hieracium*, as described and figured in the present paper, clearly originate as 'detached meristems'. This origin agrees with that described by Goebel (1905), but Koch (1893) and later Majumdar and Datta (1946) describe the origin of bud meristems from vacuolating tissue in the axils of the leaves. In the absence of more numerous investigations it is not possible to say which type of origin is most frequent in Dicotyledons. No doubt in a particular case the type of development would depend on the interval in time between leaf initiation and bud development, though nests of meristematic cells have been observed persisting in the axils of semi-mature leaves, and Flot (1906) considers that buds are initiated from apical cells however late they continue their development.

A second doubtful feature of the development of axillary buds is the origin of their traces. Again Wardlaw (1944) has provided evidence from the ferns that the traces originate basipetally, and Majumdar and Datta (1946) describe the basipetal formation of bud-traces in Dicotyledons. In the floral buds of *Dipsacus* (Philipson, 1947) and the flowering branches of *Valeriana* (Philipson, 1947*a*) the primordia originally lack any connexion with the stele of the main axis, but it was not possible to determine whether the strands which eventually make this connexion develop acropetally or basipetally. In *Hieracium*, on the other hand, the meristems of axillary buds retain lateral connexion with the edges of the leaf-gap and the mid-traces of the prophylls develop from this tissue in an acropetal direction. A possible explanation of these apparently contradictory observations may be that the vascular strands of buds that develop simultaneously with the leaves arise acropetally, whereas the

traces of buds which develop long after the leaves arise basipetally because they become seats of renewed meristematic activity isolated in vacuolated tissue. Camus refers to the formation of Cambium from cortex cells in excised buds of endive.

Thirdly, it is still a matter of debate whether the flowering branches of an inflorescence are of the same nature as the branches in the axils of foliage leaves. Grégoire (1938) and Majumdar (1945) regard inflorescence branches as fundamentally different from vegetative branches. This question has been discussed in the two preceding papers of this series, and reasons are given there for regarding inflorescence and vegetative branches as essentially similar.

#### SUMMARY

The development of the capitula of two further types of the Compositae, *Hieracium boreale* Fries and *Dahlia gracilis* Ortg., agrees with that previously described for *Bellis*, and shows the same general resemblance to that of *Dipsacus* and *Succisa*. The additional evidence provided by these types confirms the difference between the capitula of the Compositae and the *Dipsacaceae* described in earlier papers in this series, that is, the absence of meristematic tissue in the axils of the involucre bracts and often of the upper foliage leaves. The separate origin within the peripheral meristem of the primordia of the receptacular bracts and of the florets, and their early development, are identical with those of *Dipsacus*.

The axillary buds of *Hieracium boreale* remain dormant, except for the upper buds which develop into flowering branches and the basal buds which continue the rhizome. The axillary buds of *Dahlia gracilis*, on the other hand, develop into leafy branches, the apical meristems of which become committed to flowering in a basipetal sequence. The habit and life-form of these plants, therefore, is governed by the inhibition or growth of their lateral buds. Recent views on the origin and early development of lateral buds are reviewed. Additional evidence is required for the final solution of such problems as whether axillary buds originate as detached portions of the apical meristem or *de novo*, the method by which the provascular tissue of the lateral axis becomes connected to that of the main axis, and the relationship between flowering axes and their bracts.

Considerable variation exists in the vascularization of the involucre in the types of Compositae examined; the possibility of the use of these differences in the classification of the group is suggested.

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## DESCRIPTION OF PLATE II

Illustrating Dr. W. R. Philipson's article 'Studies in the Development of the Inflorescence, IV. The Capitula of *Hieracium boreale* Fries, and *Dahlia gracilis* Ortg.'

### *Hieracium boreale* Fries

FIG. 1. Longitudinal section through a stem apex in the vegetative phase  $l_1, l_2$ , leaf rudiments cut medianly;  $b_1, b_2$ , the buds in their axils. See Fig. 5 for higher magnification. ( $\times 50$ .)

FIG. 2. Longitudinal section through a stem which has become transformed into the rudiment of a capitulum. Buds,  $b_1$  and  $b_2$  in the axils of two upper leaves, the lower bud being the more advanced. ( $\times 50$ .)

FIG. 3. Longitudinal section through a stem with the terminal capitulum more advanced than in Fig. 2. Buds,  $b_1$  and  $b_2$  in the axils of two upper leaves, the upper being larger, but not yet having developed prophylls. ( $\times 50$ .)

FIG. 4. Longitudinal section through a stem in which the terminal capitulum is well developed. Of the two lateral capitula included in the section, the upper is larger and more advanced in development. ( $\times 50$ .)

FIG. 5. Higher magnification of part of the section shown in Fig. 1.  $b_1, b_2$ , axillary buds, the upper still being continuous with the apical meristem;  $d$  and  $v$ , dorsal and ventral vacuolated tissue of leaf-rudiment, separated by the provascular meristem;  $g_1$ , leaf-gap;  $p$ , adjacent periclinal divisions in the second tunica layer of the apical meristem, which indicate the initiation of a leaf. ( $\times 250$ .)

### *Dahlia gracilis* Ortg.

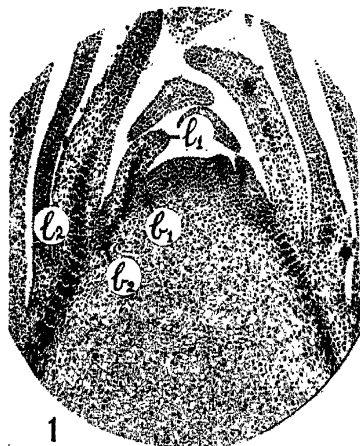
FIG. 6. Longitudinal section through a well-developed capitulum.  $i'$ , outer involucre bract;  $s$ , receptacular scale. ( $\times 11$ .)

FIG. 7. Transverse section below the terminal capitulum, of which the peduncle and portions of three involucre bracts are cut at the centre of the section.  $l_1$ , one of the uppermost pair of leaves;  $c$ , the receptacle of the capitulum of the axil of  $l_1$ , surrounded by its involucre bracts;  $a$ , a leaf produced on the axis of an aborted lateral shoot. ( $\times 12$ .)

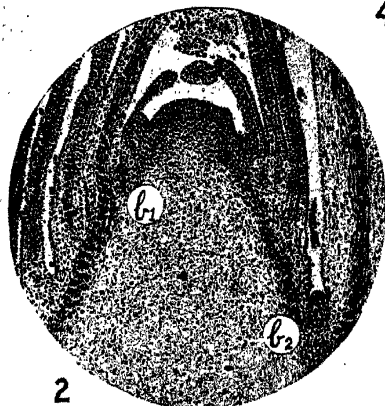
FIG. 8. Longitudinal section through a young terminal capitulum to show the disposition of meristematic tissue. The left-hand lateral bud is aborted. ( $\times 34$ .)

FIG. 9. Highly magnified portion of the meristematic mantle of a section taken from the same series as Fig. 8. The origin of a receptacular bract is shown at  $b$  where the second tunica layer has divided periclinally;  $f$ , origin of a flower primordium, the second tunica layer has divided anticlinally. ( $\times 500$ .)

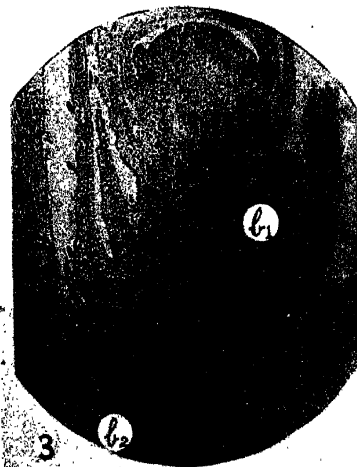
FIG. 10. Another section from the same series as Fig. 9, showing a bract ( $b$ ) and flower primordium ( $f$ ) at a later stage of development. The central provascular meristem of the bract is visible between two highly vacuolated layers. The flower primordium can be seen to have preserved the two tunica layers. ( $\times 500$ .)



1



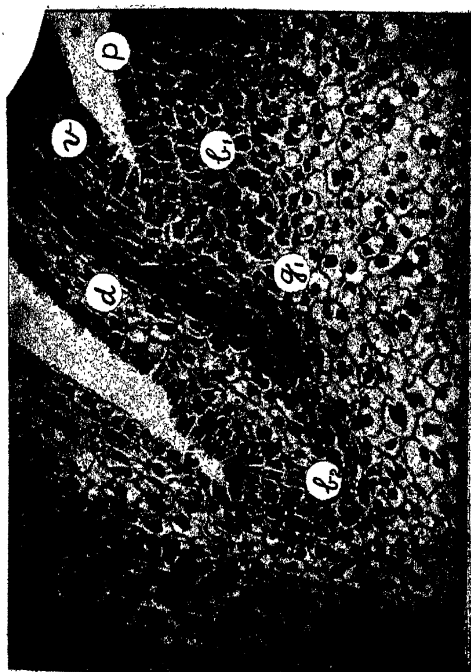
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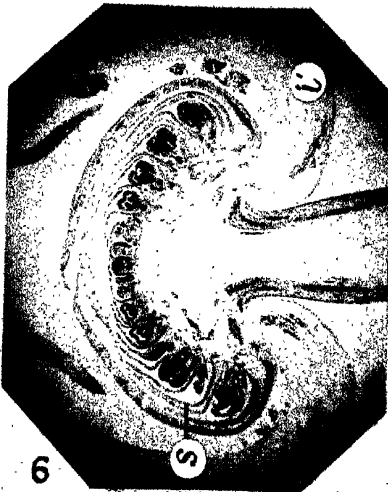
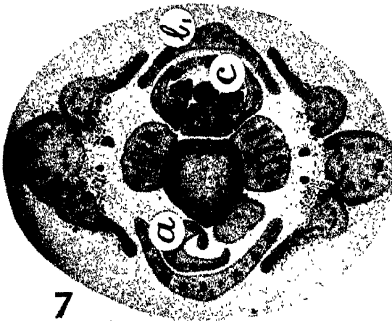
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4



5





# Stimulation of the Formation of Perithecia of *Melanospora destruens* Shear. by small Quantities of certain Phosphoric Esters of Glucose and Fructose

BY

LILIAN E. HAWKER

(Department of Botany, University of Bristol)

With one Figure in the Text

THE greatly increased production of perithecia by *Melanospora destruens* on a medium containing a relatively high concentration (5.0 per cent.) of sucrose compared with that on a similar medium containing the same amount of glucose may be in part due to the probable greater ease of phosphorylation of sucrose. Hawker (1947) showed that in this fungus fruiting was greatly increased by the addition of a small quantity of glucose-1-phosphate to either a glucose or a sucrose medium. This was in accordance with the theories of Doudoroff (1943) and Doudoroff et al. (1943) relating to the synthesis and breakdown of sucrose by bacteria by way of this ester and fructose. Hartt's (1944) work on excised leaves of sugar-cane indicates the probable importance of fructose-1:6-diphosphate as an intermediate stage in the synthesis of sucrose. This ester was not available at the time of publication of the previous paper (Hawker, 1947), but a small amount has since been obtained, together with a sample of a mixture of hexose monophosphates consisting chiefly of glucose-6-phosphate, fructose-6-phosphate and trehalose-1-phosphate. The effects of these were compared with that of glucose-1-phosphate.

The basal medium was that used in previous work, viz. medium A;  $\text{KNO}_3$ , 3.5 gm.;  $\text{KH}_2\text{PO}_4$ , 1.75 gm.;  $\text{MgSO}_4$ , 0.75 gm.; lentil extract, 0.2 gm. dry weight; distilled water, 1 litre; agar powder, 15 gm. Sugars were added to give the following concentrations: (1) 0.25 per cent. glucose plus 0.25 per cent. fructose, (2) 0.5 per cent. glucose plus 0.5 per cent. fructose, (3) 2.5 per cent. glucose plus 2.5 per cent. fructose, (4) 5.0 per cent. sucrose, (5) 0.5 per cent. sucrose. The esters were sterilized by passage through a bacterial filter and were added to the cooled media (1-4), just before the plates were poured, in amounts to give a concentration of 0.05 per cent. Owing to the limited amounts of the esters which were available medium 5 was not included in this series. For comparison a small quantity (0.05 per cent.) of sucrose was added to portions of media 2 and 3. Other plates were poured with the various modifications (1-5) of medium A, and 4 days after inoculation of these with *M. destruens* holes were cut in the agar medium and were filled with one or other of the sterilized ester solutions. All plates were incubated at 25° C.

The results of adding the esters directly to the agar medium are set out in

the table, which gives the perithecial frequency (obtained by the method first described by Asthana and Hawker, 1936) determined 14 days after inoculation. These results were confirmed by a further experiment on a smaller scale.

TABLE

*Effect of adding Hexose Phosphates to the Medium*

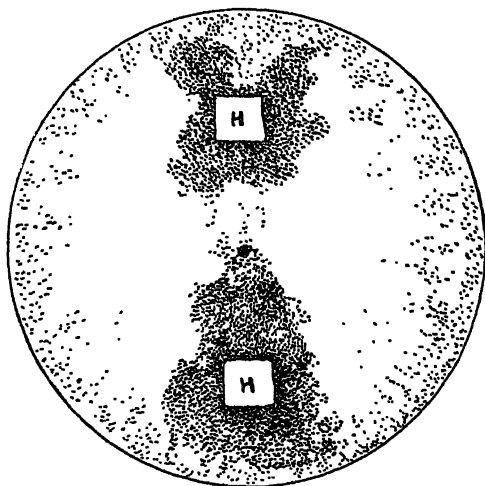
Sugars in medium.	Nature and quantity of phosphoric esters added.	Perithecial frequency 14 days after inoculation.
0.25 % glucose + 0.25 % fructose	None	4.7
" " "	0.05 % glucose-1-phosphate	6.0
" " "	0.05 % mixed monophosphates	5.4
" " "	0.05 % fructose diphosphate	6.6
0.5 % glucose + 0.5 % fructose	None	3.9
" " "	0.05 % glucose-1-phosphate	5.6
" " "	0.05 % mixed monophosphates	4.7
" " "	0.05 % fructose diphosphate	5.0
2.5 % glucose + 2.5 % fructose	None	few
" " "	0.05 % glucose-1-phosphate	4.9
" " "	0.05 % mixed monophosphates	4.9
" " "	0.05 % fructose diphosphate	3.4
5.0 % sucrose	None	5.4
"	0.05 % glucose-1-phosphate	8.8
"	0.05 % mixed monophosphates	7.9
"	0.05 % fructose diphosphate	8.4

Small amounts of any of the three preparations thus caused a considerable increase in fruiting with either sucrose or glucose and fructose media. The addition of small amounts of sucrose to media containing a relatively large quantity of hexose sugar increased fruiting but was not so effective as the addition of the phosphoric esters.

Where holes cut in the medium were filled with the test solutions a zone of dense fruiting developed round the holes (see text-figure).

These results were not due to growth-substances present as impurities in the preparations of the esters since the medium already contained a sufficient amount of these in the form of lentil extract. Moreover, no stimulatory effect followed when similar holes were filled with the lentil extract or with a solution of pure vitamin B<sub>1</sub>.

One must, therefore, conclude that the phosphoric esters of hexose sugars are active in promoting fruiting of this fungus. The significance of the effect of glucose-1-phosphate has been discussed in the earlier paper (1947). Since pure fructose-1 : 6-diphosphate or a mixture of monophosphates are about as active as glucose-1-phosphate, it is assumed that once phosphorylation has taken place interconversion of one ester to another occurs readily. The actual path by which glucose and sucrose are broken down by *M. destruens* therefore remains obscure. The evidence suggests, however, that the ease of phosphorylation is an important factor in determining the effect of a particular sugar on fruiting.



Drawing from a photograph of a 14-day-old culture of *M. destruens* on medium A containing 0.5 per cent. glucose plus 0.5 per cent. fructose. Holes (H) cut in the medium were filled with a solution of glucose-1-phosphate 4 days after inoculation. The closeness of the dots represents the distribution of perithecia but a dot may represent more than one perithecium in the densely crowded zone immediately surrounding the holes.

#### SUMMARY

The production of perithecia by *M. destruens* on a synthetic medium containing an adequate supply of growth substances and with either sucrose or a mixture of glucose and fructose as the source of carbon is stimulated by the addition to the medium of 0.05 per cent. of various hexose phosphates. Since the stimulating effects of glucose-1-phosphate, of a mixed sample of monophosphates and of fructose-1:6-diphosphate are approximately equal it is concluded that once phosphorylation has taken place the various hexose phosphates are readily interconvertible.

The writer's thanks are due to her colleague Dr. E. W. Yemm who prepared the fructose diphosphate from beer yeast and supplied the sample of mixed monophosphates, and to Dr. H. Porter for the sample of glucose-1-phosphate.

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# Physiological Studies on Nodule Formation

## I. The Relation between Nodulation and Lateral Root Formation in Red Clover

BY

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With four Figures in the Text

### INTRODUCTION

LEGUMINOUS root nodules and lateral roots were at one time considered to be homologous organs on the grounds of a number of morphological and anatomical resemblances, but the demonstration that a nodule is in fact a metamorphosed lateral root in development must rest on more than simple observation. It is well known that the penetration of the infection thread through the cortex takes place contemporaneously with and not subsequent to the development of the pericycle or cortical cells into the initial nodular tissue. This has been elegantly demonstrated by Wipf and Cooper (1940) in cytological preparations of young infected roots which show the tetraploid cells, later to become nodular tissue, in division in advance of the primary infection thread. The closest examination fails to establish whether the penetration of the slime thread from an infected root-hair into the cortex of the root is a consequence of meristematic activity in the root or whether cell division follows immediately upon penetration. The lack of evidence for the former sequence of events is no proof that the plant does not in fact play an important role in limiting the number of infections, since it is possible that the infection thread will grow into the cortex from the infected root-hair only if some cells in the neighbourhood are predisposed to meristematic development.

These opposing views on infection may be briefly formulated as follows: (1) The successful penetration of the infection thread stimulates the cells of the root to divide, the morphology of the resulting nodule being merely a consequence of the realized potentialities of any root meristem; or (2) the infection thread in the root-hair is able to penetrate the cortex only at points of incipient meristematic activity, i.e. the foci of infection are predetermined in the plant, possibly at the site of lateral root initials.

The essential difference between these two alternatives rests upon whether the initiation of the process of nodule formation is determined by the invading organism or whether a determinate role is played by the plant. In the former case the relations would be explained in terms of virulence or infectivity of the bacteria, in the latter in terms of susceptibility or resistance of the host tissues. There is sometimes a confusion of these two modes of approach, and in the present paper both terms have been avoided.

The first view is the one most generally held, though it may be said that all evidence from studies on the processes of infection, development of nodules, &c., may be interpreted to conform with either hypothesis.

The following anatomical considerations are generally considered to weigh against a modified root hypothesis of nodule development. Lateral roots invariably arise in the primary root by the division of pericycle cells, whereas the primary cell divisions leading to nodule formation seem less well defined in position; they may arise not only in the pericycle but also in the inner layers of the cortex, and in the case of the bean (Prazmowski, 1890) in the outer layers as well. Also, the nodule lacks a root-cap and has a peripheral instead of axial vascular system, the different strands of which may arise from diverse primary xylem elements.

Taken together these considerations are cogent, but they rest on no invariable generalizations with respect to root structure, as the following facts show. First, roots arising on the shoot may have either endogenous or exogenous origin, and lateral roots developing on primary roots with secondary thickening develop from tissues other than the pericycle. Second, root-cap development in the Spermatophytes shows every gradation from the complete separation of a calyptragen from the meristem in, for example, the Gramineae, to an ill-defined root-cap in, for example, *Pisum*, or to a total absence of root-cap in a large number of aquatic plants, in some Coniferae, in some parasitic plants, and so on. Third, the peripheral disposition of the vascular traces is usual, but not invariable, in nodules. Spratt (1919) showed that in *Lupinus* sp. the vascular strand is axial at the base of the nodule, and branches distally, the bacterial tissue being located within and between the vascular strands. Brenchley and Thornton (1925) have shown that in the mature nodule the bacterial tissue and vascular strands are enclosed within an endodermis continuous with that of the root, so that all the nodule tissues except the rind are at maturity located within the outermost cell-layer of the original plerome.

There does not appear, therefore, to be any ultimate anatomical objection to regarding nodules and lateral roots as homologous structures; nodules merely display a number of otherwise rather exceptional features of root structure and development.

In the experiments to be described a new approach is made to the analysis of the factors limiting the number of infections occurring on the root; this consists in an examination of the development of both lateral roots and of nodules on plants inoculated and initially uninoculated, employing strains of clover known to differ in the number of nodules formed under standardized conditions. These strains of clover have been developed in the course of genetical researches relating to nodule number, but these researches need not be fully dealt with in this paper (Nutman, 1946a).

#### MATERIAL AND METHODS

In all experiments the clover plants were grown on slopes of a mineral salts agar medium in test-tubes under sterile conditions, and inoculated from

a slope culture of nodule bacteria. The composition of the nitrogen-free agar medium used, and the technique of seed sterilization, have been previously described (Thornton, 1930), and the effective and ineffective strains of nodule bacteria employed as inocula are named strain A and strain H.K.C. respectively; both are known to be fairly stable as regards the number and effectiveness of nodules produced on normal plants (Nutman, 1946b).

TABLE I  
*Origin of Families of Plants*

Parent plants		Sparse types.				Abundant types.			
♂	♀	m	n	o	u	k	q	S'b	S'e
Sparse types	m		s × s		s × s	a × s	a × s		
	n	s × s		s × s		a × s			
	o		s × s						
	u	s × s							
Abundant types	k	s × a	s × a				a × a	a × a	a × a
	q	s × a				a × a			
	S'b					a × a			
	S'e					a × a			

s < s, sparse types; s × a, mixed types; a × a, abundant types.

The lines of clover used were crosses between first selections, on the basis of the number of nodules produced, from commercial late-flowering Montgomeryshire Red Clover and from the Aberystwyth strain S151 (i). Crosses were made between the plants designated 'm', 'n', 'o', and 'u' originally selected as forming few nodules (sparse types), and the plants 'k', 'q', 'S151 (i) b', and 'S151 (i) e' selected as forming many nodules (abundant types). The families employed in the following studies were: m × n, m × u, and n × o and reciprocals, i.e. crosses between 'sparse types'; k × q, k × S151 (i) e and k × S151 (i) b, crosses between 'abundant types' and k × n, k × m, and q × m, crosses between sparse and abundant types which produced an intermediate number of nodules. The origin of these families is possibly made clearer by reference to Table I.

## EXPERIMENTAL RESULTS

A preliminary experiment was carried out in 1943 utilizing the crosses  $m \times n$ , forming few nodules, and  $m \times k$  and  $n \times k$ , forming an intermediate number of nodules. The plants were sown singly on slopes of a standard medium and the cultures remained at first uninoculated. At intervals the

KEY: ● Family  $m \times n$  (Sparse type)  
 ○ Family  $n \times k$  (Mixed type)  
 ◐ Family  $m \times k$  (Mixed type)

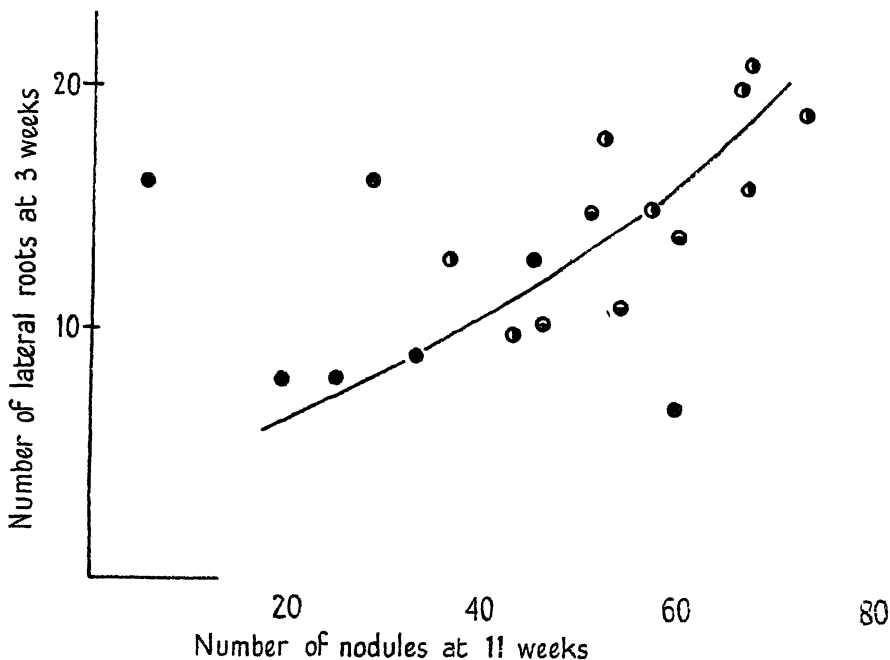


FIG. 1. The relation between the number of lateral roots formed in red clover before inoculation to the number of nodules which are afterwards produced.

number of lateral roots was counted on each plant until the crowding of the roots at the base of the tube made accurate counts impossible. The plant cultures were then inoculated (at 6 weeks old) with an effective strain of bacteria (strain A) and allowed to grow for 4 months, when the numbers of nodules at harvest were determined. The relation between the number of lateral roots at 3 weeks and the number of nodules at 11 weeks is shown in Fig. 1, in which there appears to be a linear relation between the number of lateral roots produced on seedlings before inoculation and the number of nodules which were afterwards formed. Three points in the diagram are very aberrant.

This result may be interpreted in one of two ways: either (1) that the plants eventually forming the larger number of nodules produce a more extensively branching root system, thus having a greater total length of root exposed to random infection; or (2) that the lateral root initials of such plants provide a larger number of potential foci of infection capable of developing into nodules. On the first view the limitation of nodule number will depend solely upon factors determining the effective surface area of the root exposed to infection, and this will itself be dependent upon the number and length of lateral roots formed. Interpretation thus demands knowledge of the factors controlling lateral root formation and root growth (other than the genetical factors mentioned above), and of the effects of nodulation on lateral root formation.

#### DEVELOPMENT OF NODULES AND LATERAL ROOTS

In the following year a more comprehensive experiment was carried out using the nine hybrid families of plant material enumerated above. One complete series of nine families, each family comprising about one dozen plants, was inoculated at the outset with the effective strain (strain A); a second series was inoculated with the same strain after the mode of branching of the uninoculated root had been ascertained; and a third incomplete series of the crosses  $m \times n$ ,  $m \times q$ ,  $q \times k$  was inoculated at the outset with the ineffective strain (strain H.K.C.).

Care was taken in setting up the experiment to include only those culture tubes in which the plant germinated immediately after sowing, and in view of the known influence of the volume of root medium on the number of nodules produced (Nutman, 1945), this was standardized and later controlled by careful watering.

In the preliminary experiment the heavy deposit of phosphate at the base of the tube obstructed the view and curtailed the counts of lateral roots. This difficulty was partially overcome by allowing the heavier precipitate from the culture solution to settle before the agar was added to the decanted supernatant liquid, though even in the clear medium observations could only be made of the number of lateral roots and number of nodules present on each plant at intervals to the end of the first 6 weeks of seedling growth owing to the crowding of roots at the base of the tube. At the end of the experiment the final numbers of nodules formed were counted, and in addition in a limited selection of the treatments the final numbers and lengths of all the lateral roots were determined. The inoculated treatments were harvested after about 4 months' growth, and those initially uninoculated about 6 months after being sown.

The results of the lateral root and nodule counts made during seedling development are summarized on the chart (Fig. 2), in which the behaviour of the nine families of the plant material are compared with respect to lateral root formation and nodule production, when uninoculated, or inoculated with effective and ineffective strains of bacteria respectively.

The diagrams in Fig. 2 constitute two rows showing the behaviour after inoculation with effective and ineffective strains respectively, and three columns showing the behaviour of the three plant types used, i.e. the crosses sparse  $\times$  sparse, abundant  $\times$  abundant, and mixed types (sparse  $\times$  abundant). The curves in the diagrams show the increase in the number of lateral roots in

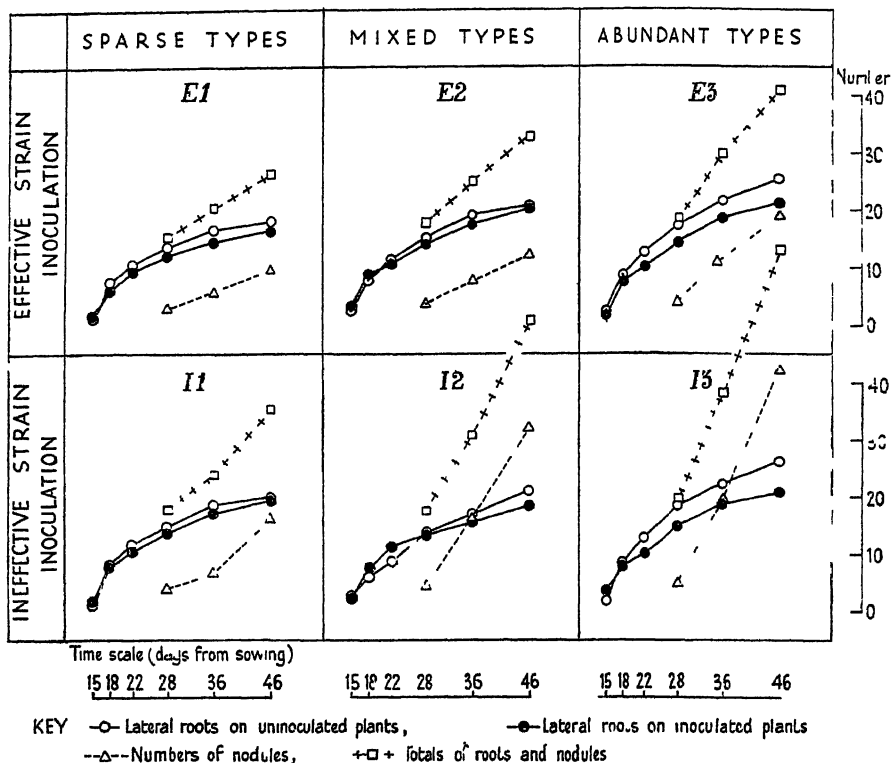


FIG. 2. The average numbers of lateral roots and nodules formed by the progeny of crosses between parent plants of red clover selected for high and low nodule number E1, the families  $m \times n$ ,  $m \times o$ , and  $m \times u$  (29 plants inoculated, 27 plants uninoculated); E2,  $n \times k$ ,  $m \times k$ , and  $m \times q$  (28 inoculated, 29 uninoculated); E3,  $k \times q$ ,  $k \times S'b$ , and  $k \times S'e$  (33 inoculated, 30 uninoculated). I1, the family  $m \times u$  (14 plants inoculated, 11 plants uninoculated); I2,  $m \times q$  (11 inoculated, 11 uninoculated); I3,  $q \times k$  (13 inoculated, 12 uninoculated).

uninoculated plants (open circles) and inoculated (black circles). The nodule numbers are shown by a broken line and the totals of lateral roots and nodules indicated by crosses. The following results are apparent. (1) The number of lateral roots produced increases from the sparse  $\times$  sparse crosses to the abundant  $\times$  abundant crosses. This effect is more marked in the early stages of development; the final number of laterals differs much less, particularly so in the plants with ineffective inoculation. (2) The number of laterals is consistently greater in the uninoculated plants, indicating a possible inhibition of lateral root formation by the presence of nodule bacteria in the medium.

(3) A similar trend of increase from sparse to abundant types is seen even more clearly in the number of nodules produced. This effect is much greater with ineffective inoculation. In the effective strain the number of nodules produced up to 46 days from sowing is always less than the number of lateral roots, but in sets inoculated with the ineffective strain nodule number exceeds lateral roots soon after nodulation begins. The difference already noted in the progress of lateral root formation as between effective and ineffective strains among the plant types is no doubt associated with this fact. (4) The sum total of laterals and nodules after nodulation begins follows a nearly linear course with ineffective strains and shows a tendency to fall off in the effective.

These results based on average performance of the different combinations of plant and bacterial type indicate that some general factor is concerned controlling both lateral root formation and nodulation. The differences between the plant types demonstrate that this factor is internal to the plants. The slope of the line showing the sum of nodules and laterals is clearly related also to the initial rate of lateral production before nodulation begins. The cause of the falling rate of lateral production may probably be attributed to the conditions under which the plants were grown and more particularly to the limitation of leaf development. This is not a matter of concern in this paper, but the curvilinear nature of the relation makes direct comparison of the rates difficult. By plotting the numbers against the logarithm of time after planting the curves approach straight lines, and use was made of this relation for the estimation of linear regression coefficients between numbers of nodules and lateral roots and the logarithm of time from sowing for the comparison of the behaviour of the plant types and of individual plant families.

These regression coefficients are entered in Table II, where data for individual families are presented both for plants inoculated at sowing with the effective (strain A) and ineffective (strain H.K.C.) bacteria, and those left for about 6 weeks before inoculation (with strain A). Average values for the various plant types inoculated at once and after delay are also entered.

The regression coefficients based on all the data indicate that the final nodule numbers, at the end of 6 months, for the different plant types is related to the rate of lateral production during the first 6 weeks in the delayed inoculation series. In the series inoculated with the effective strain A both the rate of lateral production and the rate of nodulation estimated over the first 6 weeks fall into the same order as the final nodule number at the end of the experiment.

These findings confirm the suggestion of a controlling factor associated with plant type and affecting both the rate of lateral root production and the rate of nodulation. When the data for the individual families are considered a similar relationship is indicated.

The data for the average value of the three plant types plotted on a logarithmic scale of time for the rate of lateral root production and rate of nodulation are again presented in Fig. 3, which refers only to plants inoculated with the effective strain. This figure brings out clearly the linear relation

TABLE II

*Average Rates of Nodule and Lateral Root Formation, and Mean Total Numbers of Nodules per Treatment*

s × s 'sparse types' forming few nodules; s × a 'mixed types'; a × a 'abundant types' forming many nodules

		Inoculum (initial).	Number of replicates.	Regression coefficients. (a) No. of lateral roots.	(b) No. of nodules.	Mean no. of nodules at harvest.
	Cross.					
s × s	m × n	O	7	28.3	—	25.5*
	m × o	"	9	30.6	—	27.7*
s × a	k × m	"	6	44.5	—	42.8*
	k × n	"	12	34.0	—	36.5*
a × a	k × s'b	"	8	38.5	—	47.3*
	k × s'e	"	10	60.5	—	77.9*
s × s	m × n	Strain A	7	19.7	34.8	17.1
	m × o	"	9	22.6	32.3	18.7
s × a	k × m	"	5	39.4	37.9	21.2
	k × n	"	12	36.4	34.3	25.3
a × a	k × s'b	"	8	41.5	70.0	50.4
	k × s'e	"	12	32.9	72.3	42.1
s × s	m × u	O	11	35.6	—	32.1*
s × a	q × m	"	11	36.6	—	39.2*
a × a	k × q	"	12	47.0	—	89.2*
s × s	m × u	Strain A	13	37.4	17.0	16.6
s × a	q × m	"	11	25.1	35.2	24.6
a × a	k × q	"	13	37.0	53.7	40.7
s × s	m × u	Strain H.K.C.	14	33.7	66.8	52.6
s × a	q × m	"	11	29.5	133.7	96.0
a × a	k × q	"	13	34.6	174.7	130.4
Average s × s	O		27	32.0	—	29.0*
" s × a	"		29	36.3	—	38.8*
" a × a	"		30	44.3	—	74.3*
Average s × s	Strain A		29	28.6	26.3	17.4
" s × a	"		28	32.6	35.2	24.3
" a × a	"		33	37.9	68.5	43.6

\* These plants were inoculated 6 weeks after sowing.

indicated, and extends the data for nodulation to a later date. The varying slopes of the lines on which the regression coefficients already presented were based appear very clearly, and it is seen that the effect of plant type on nodulation extends up to 126 days from sowing, at which time the main part of the experiment was terminated.

Three other points are inserted on the diagram representing the number of nodules found 6 months from sowing on sets of plants of the three categories in which inoculation was delayed until 6 weeks from planting. These points have been joined by dotted lines to the final values of nodule number obtained after 126 days by plants inoculated at sowing. It will be noted that these construction lines converge to a point on the abscissa not far removed from the arrow representing the time of inoculation of the delayed sets. The slopes of these curves may thus be regarded as representing the rates of

nodulation, and these are seen to be for corresponding sets greater than the rates for plants inoculated at sowing. Although the lines inserted do not strictly display nodulation rates for the delayed plants, the fact that they con-

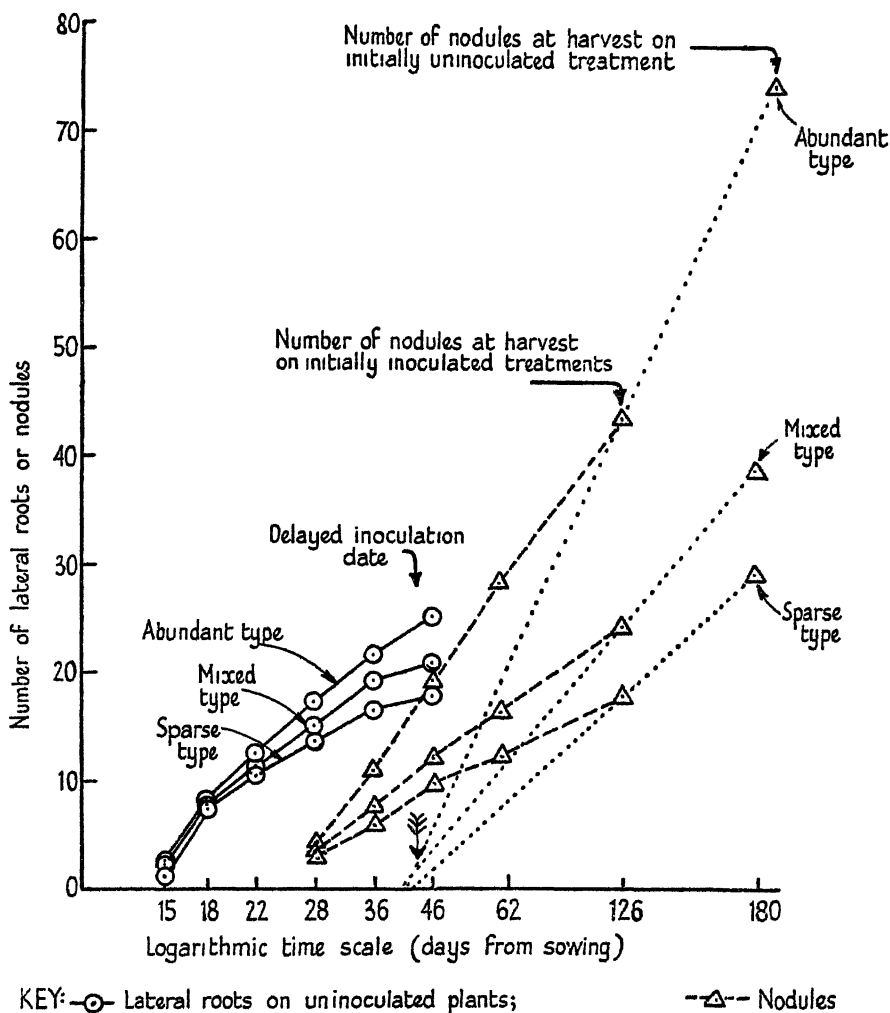


FIG. 3. Average numbers of lateral roots and nodules on three selected lines of clover inoculated with an effective strain either at planting or after a delay of six weeks.

verge to a common point indicates that the rate of nodulation after delayed inoculation is for all three types of plant proportional to the rate obtained with immediate inoculation. This again shows that an internal factor characteristic of the plant type persists over long periods of time. The new salient point, however, is that delay in inoculation increases the subsequent rate of nodule production.

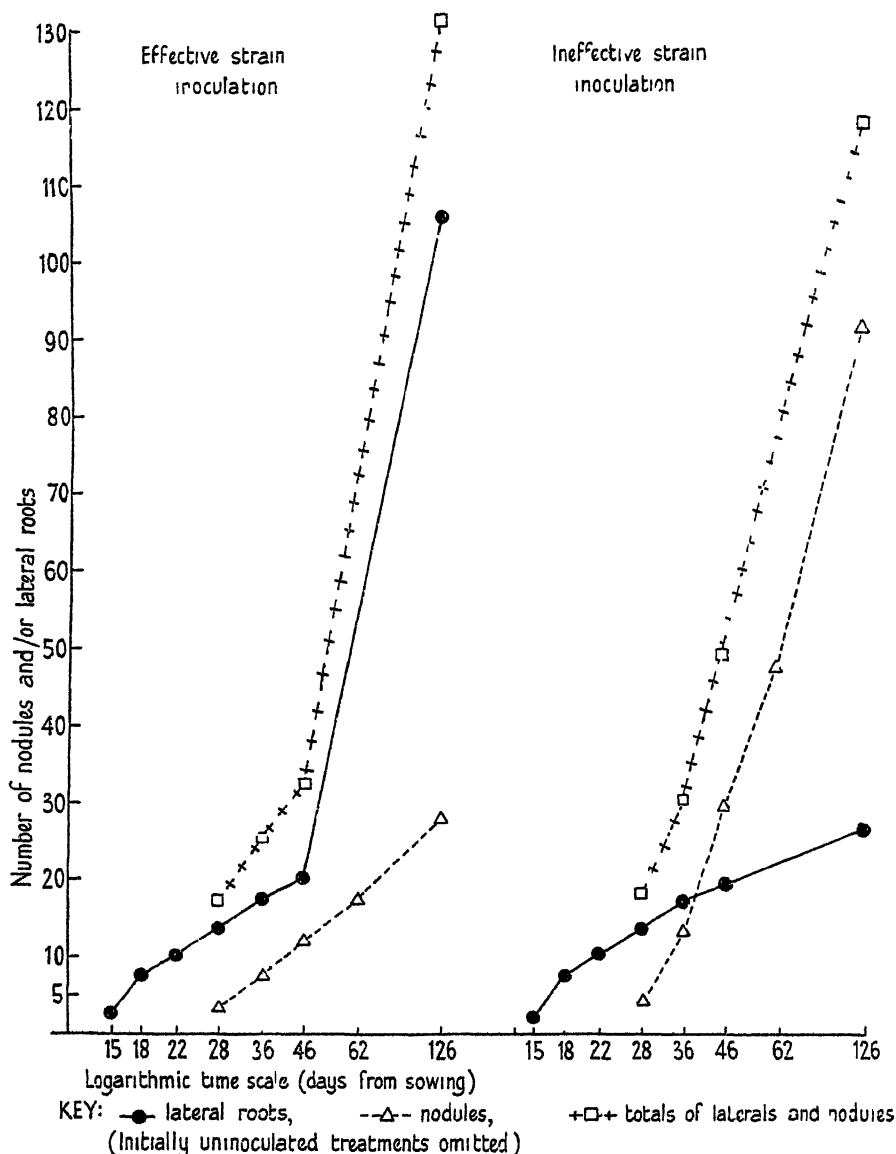


FIG. 4. Average numbers of lateral roots and nodules on all three selected lines of clover with effective or with ineffective strain inoculation.

A direct comparison between the effects of the two bacterial strains on the rate of lateral root production, nodule formation, and totals of roots and nodules is presented in Fig. 4. In this diagram, again plotted on a logarithmic time scale, the three plant types are not separated, but the average values for all the data available are set out. The sum total of lateral roots and nodules

produced by the two strains shows comparatively small difference in the rate of increase due to the two strains. The effective strain at first shows a lower total, but at the end of 126 days the order is reversed, the effective strain giving the larger total. If, however, the number of nodules alone is considered it is seen that from the outset of nodulation the ineffective strain inoculation has produced a much more rapid nodulation, and the characteristic rates for the two strains remain sensibly constant. Lateral root production up to 6 weeks from sowing is unaffected, but in the subsequent 80 days the lateral root production of the plants inoculated with the effective strain has increased very greatly, whereas in those inoculated with the ineffective strain the rate of lateral production remains almost the same as before.

The results suggest that the differences in the number of nodules produced by the effective and ineffective strains is associated with a different behaviour of the initials. On the hypothesis examined in this paper, namely, that nodules are metamorphosed roots, the results would indicate that some initials which can produce nodules with an ineffective strain grow out as lateral roots in the presence of an effective strain. The sum total of laterals plus nodules as seen in the diagram does not differ greatly with the two strains, and this may be interpreted as showing that the total number of foci from which either roots or nodules can arise is limited, and that nodule number characteristic of the two strains arises from this differing behaviour. So far the evidence is based on rather slender data; the hypothesis is supported but not proved.

It should also be emphasized that these conclusions are based on average values, and the examination of the results of individual plants from families of varying genetic constitution show great variation in the degree of association of the developmental factors. It will be the task in subsequent work to analyse these individual differences between plants. It should be noted that the parents of the hybrid families used were derived by selection from a commercial strain of red clover and were in no sense pure lines.

The higher nodulation rate in the abundant  $\times$  abundant crosses than in the other two types might be attributed to a larger root system obtained in these plants owing to a higher rate of lateral formation. Each lateral as it develops would afford a greater surface area through which the invasion of bacteria can take place. To examine this possibility the total length of the root system at harvest in a random selection of individual plants was compared with the number of nodules formed, so that the density of nodules per unit length of root was ascertained.

In Table III such data are presented for the various plant types inoculated with the effective and ineffective strains. The great increase in size of the root system of the effective inoculated set as compared with the ineffective is the most striking feature of the table. It will be noted that the number of nodules per metre of root varies very greatly, but beyond the fact that with the ineffective strain the density is uniformly higher, little regularity can be seen.

TABLE III

*The Total Length of Root and Final Number of Nodules of Individual Plants*

	Family.	Total length in mm. of roots at harvest.	Number of nodules at harvest.	Number of nodules per metre root.
<i>Inoculated with effective strain A</i>				
Sparse types	m × u	1,850	23	12.4
	"	765	15	19.6
	"	1,331	10	7.5
	"	1,032	20	19.4
	u × m	1,632	15	9.3
	"	2,328	27	11.6
	"	1,666	29	17.4
	"	960	15	15.6
Mixed types	"	1,008	5	5.0
	k × n	1,215	31	25.5
	n × k	2,692	7	2.6
Abundant types	k × q	1,626	22	13.5
	"	2,103	71	33.8
	q × k	2,206	25	11.3
	"	2,042	57	27.9
<i>Inoculated with ineffective strain H.K.C.</i>				
Sparse types	m × u	448	26	58.1
	"	330	52	157.5
	"	398	67	168.4
	"	176	6	342.5
	u × m	490	73	146.8
	"	434	25	58.0
Mixed types	"	466	75	161.0
	m × q	512	51	100.0
	"	421	50	118.8
	"	385	76	197.2
	q × m	802	162	202.0
	"	456	86	188.7
Abundant types	"	428	83	193.8
	k × q	505	125	247.5
	"	508	142	279.3
	"	615	91	147.9
	q × k	663	116	174.8
	"	558	96	172.1
	"	465	150	322.6
	"	627	224	357.1

Within the strains no consistent differences are found. The important fact is that the values of density in no way tend to a constant value, so that the evidence is against the view that the size of the root system is an important factor.

### DISCUSSION

Although a great deal of research has been devoted to the study of the mode of origin and the development of the individual nodule, the varying efficiencies of bacterial strains in inducing nodulation and the functional activity of the bacteria, it is surprising that the part played by the plant in the symbiosis has

hitherto been neglected. Plant roots are exposed to mass infection, and McCoy (1932) has emphasized the widespread infection of the root-hairs. Nevertheless the number of nodules produced on the plant is very small compared with the number of points of invasion. If nodules were purely adventitious in origin there seems no reason, *a priori*, why the whole of the root system should not be densely covered with nodules, which manifestly is not the case. It is, however, evident from the behaviour of effective and ineffective strains that the degree of development of nodules can vary within wide limits. Considerations of these strain differences and of the relationships which have been shown to hold between lateral root and nodule development have led to the view that there are two sets of factors dependent upon the plant reaction which determine the distribution and numbers of the nodules produced. Provisionally these are considered to be (1) a limitation of points of invasion and of the possible foci of nodule formation, (2) a morphogenic effect of pre-existent nodules of the behaviour of the other initials from which nodules may arise. The word 'initial' may misleadingly suggest a visible meristematic group of cells. It is not intended to convey this, but rather some stage prior to any visible meristematic activity. The observations of Wipf and Cooper (1940) have shown that nodule formation is associated with specific polyploid cells which, as they state, are often found near root initials, and it is to some stage of development of this kind prior to any meristematic activity that the word 'initial' is applied.

(1) The evidence presented in this paper supports the view that the possible foci of infection are the actual initials from which lateral roots arise. The sum total of roots and nodules has been shown to be almost constant in plants inoculated with either effective or ineffective strains of bacteria. The variations between plant types in regard to the number of nodules formed has been shown to be clearly related to the number of such lateral root initials characteristic of the plant types. It is interesting to note in this connexion that in the early stages of nodulation, nodules may actually arise in the axils of lateral roots as though the initial has branched to produce a lateral root and a nodule primordium.

One of the difficulties in accepting this view is that teleologically speaking conversion of every lateral initial into a nodule would altogether restrict the development of the root system, and therefore the association just noted of laterals and nodules at the same focus may be regarded as advantageous as securing both early nodulation and rapid expansion of the root system. From the same teleological point of view a restriction of the development of lateral initials into nodules is an advantage to the plant; some mechanism may be looked for to secure such a restriction to ensure further development of the plant.

(2) A method by means of which such restriction of nodulation might be controlled has already been suggested, namely, an inhibiting effect of mature nodules on either the conversion of later-formed foci from lateral roots into nodules or merely on their actual initiation. It is suggestive (Chen and

Thornton, 1940) that with ineffective strains the development of the nodule is never complete but is arrested by cessation of growth of the apical meristem accompanied by the degeneration of the bacterial tissue. This strongly suggests that the inhibiting substance originates either in the apical meristem or in the bacterial tissues. A suggestion of such inhibitory action of some part of the nodulated root system has elsewhere been presented as an interpretation for the effect of the volume of the root medium (Nutman, 1945), and work has been carried forward to isolate such an inhibitory substance. Both these observations will be dealt with in later papers of this series. Much remains to be done in this connexion, for obviously a study of the number and origin of lateral root initials in plants characterized by varying nodule numbers will require to be undertaken.

An attempt has been made by Thimann (1936) to interpret nodulation in terms of the initiation and infection of lateral root primordia. He regards as an important factor the production of  $\beta$ -indolylacetic acid by the bacteria, which in very low concentrations he holds to stimulate lateral root production. Since he agrees with the present writer that the nodule is a metamorphosed root, the bacteria would induce the formation of the initials. Reference to Fig. 1 will disclose, however, that in the presence of the bacteria lateral root production is reduced, even before nodulation begins; and although the production of indolylacetic acid by the bacteria, and in larger amounts by the ineffective strains, has been confirmed, it seems doubtful that this will account for the larger number of nodules produced by the ineffective inoculations. On the contrary, the mere fact that the number of initials (nodules plus laterals) is independent of the effectivity of the strain argues against such a view.

Even these considerations do not exhaust the various modes of control exerted by the plant. The fact that plants have been isolated from a mixed population which are absolutely resistant to nodulation (Nutman, 1946a), although of course they freely produce lateral roots, indicates that absence of nodulation can occur independently of the nodule meristem.

To this nexus of problems the whole question of cross-inoculation group specificity also belongs, and it may be suggested provisionally that inhibitors of nodulation may be associated with the apical meristem of the root.

The analysis promises to be difficult in view of the fact that in normal course of development nodules and lateral roots are produced simultaneously by the plant. This may arise either by a limitation of the range of action of a nodule or by quantitative variation in the rate of production of the inhibitor or may be merely a result of the fact that the zone of root-hairs through which invasion takes place is of limited extent owing to the degeneration of the root-hairs themselves.

As to the origin of the initials, at present very little is known, so that it is unprofitable at this stage to discuss the matter further. Factors which may be concerned are the rate of transport of inhibitory substances, the possible irreversibility of development in the initial after a certain stage has been reached, and the critical levels of the inhibitor leading to either complete

suppression of lateral formation or nodulation. Admittedly these are speculations, but they have been put forward to outline the direction in which the analysis will be pursued.

Finally, the interesting mode of increase of nodules and lateral roots and the relation between them throws some light upon the problem of the immunity which the plant has been supposed to develop to further infection, as first suggested by Süchting (1904). The above results have shown that the eventual limitation is inherent in the plant, and that it operates from the outset of nodulation. The possibility of an immunity relationship or that substances produced by the root or nodule influence nodulation is not excluded, but the suggestion in this paper is that a secondary limitation of this kind is due to the production of inhibitory substances in the nodule meristem, an activity not necessarily a result of infection but which may be a normal function of any root meristem.

#### SUMMARY

The following results were obtained in a comparative study of the formation of lateral roots and nodules on red clover grown in test-tubes on slopes of an agar medium, the plant material consisting of the progeny of parent plants selected for high and low nodule number.

1. The rate of formation of nodules and of lateral roots was determined by the same hereditary factors in the plant.

2. With both effective and ineffective bacterial strains the rate of nodulation was highest at the beginning and decreased subsequently, the rate relative to the logarithm of the age of the plant being constant.

3. After the initial stage of development plants inoculated with an effective strain developed lateral roots more rapidly than plants inoculated with an ineffective strain, the sum of lateral roots and nodules being approximately the same at harvest.

4. A high rate of lateral root initiation was not associated with the formation of a more extensive root system, so that the number of nodules formed is not dependent upon the size of the root system open to infection.

5. Seedlings inoculated with effective or ineffective strains of bacteria had at first fewer lateral roots than corresponding uninoculated seedlings.

6. Delay in inoculation led to an enhanced rate of formation of nodules.

7. From these results it is concluded that lateral roots and nodules are physiologically homologous, and that infection by the bacteria can only take place at predetermined foci on the root corresponding to the sites of initiation of lateral roots.

8. It is suggested that the simultaneous appearance of nodules and lateral roots on the inoculated root may be accounted for by postulating a further restriction of infection to those foci which arise in the zones of the root on which growing root hairs are to be found, and that strain differences may be due to the production in the nodule meristem of inhibitory substances.

The author has much pleasure in recording his indebtedness to Prof. F. G. Gregory, F.R.S., whose searching criticism of current theories of nodulation led to the exploration of the alternative possibilities set out in this paper, and for his sustained interest during the progress of the work; also to Dr. H. G. Thornton, F.R.S., whose wide knowledge in this field and guidance and encouragement have been of great value. Thanks are also due to Miss Joan Crawley and Miss Mabel Dunkley for able technical assistance in the carrying out of this work.

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# Experimental and Analytical Studies of Pteridophytes

## XI. Preliminary Observations on Tensile Stress as a Factor in Fern Phyllotaxis

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With Plate III and four Figures in the Text

### INTRODUCTION

IN this paper experimental observations are set out which contribute to our knowledge of phyllotaxis in ferns; but a full consideration of this complex subject must be reserved until later. These observations relate to the induction of tangential tensile stress in the shoot apex as a result of the growth of the young leaf-primordia. The view is advanced that such stresses constitute one of the factors which determine the position of new leaf-primordia.

During the formation of the individual leaf-primordium in a fern such as *Dryopteris aristata*, a conspicuous enlargement of the basal region takes place, particularly in the tangential direction. This is mainly due to the development of parenchyma in the pith and cortex. In a very young primordium, as seen in transverse section, the incipient vascular tissue consists of an uninterrupted crescent of small-celled tissue. The growth of this tissue does not keep pace with the growth of the pith, and it becomes broken up into a number of separate strands or meristeles, i.e. apparently as a result of the tangential stress imposed by the pith or jointly by the pith and cortex (Wardlaw, 1944, 1945). The incipient vascular tissue in the regions of disruption undergoes a parenchymatous development; in other words, potential phloem and xylem are transformed into parenchyma. Experimental evidence of this phenomenon has also been obtained (Wardlaw, 1947). In relation to these developments in the leaf-primordia, the incipient shoot-stele, which initially consists of an uninterrupted cylinder, is also subjected to tensile stress in the regions of insertion of the leaf-traces, and parenchymatous leaf-gaps develop. The adult stele thus consists of an open vascular meshwork or dictyostele. These conceptions of stelar morphology in ferns were based on anatomical studies of development at the shoot apex. Experimental proof that leaf-gaps are due to the enlargement of the leaf-bases was sought by destroying leaf-primordia at a very early stage: by this procedure the development of local stresses would be avoided and the vascular cylinder would remain uninterrupted, i.e. solenostelic. When the appropriate experiment was carried out the result predicted was obtained (Wardlaw, 1944).

Such observations suggest that at the apical meristem of ferns—a conical

structure on which leaf-primordia are formed as superficial outgrowths—the development of tensile stresses may be important in morphogenetic processes. The initial hypothesis now advanced is that *as each leaf-primordium enlarges it sets up a tangential tensile stress in a localized region of the apical meristem*, i.e. immediately above the leaf axil. A further hypothesis is that *the latest primordium to be formed is situated in that region of the meristem in which tensile stress is minimal*. These hypotheses, which have resulted independently from the writer's anatomical studies of leaf development, closely resemble the mechanical explanation of phyllotaxis advanced by Hofmeister in his 'Allgemeine Morphologie der Gewächse' (1868, p. 508). Already M. and R. Snow (1931, 1933, 1947, 1947a) have made valuable experimental observations bearing on this topic. In contrast to the theoretical views of Schuepp (1914, 1916) and Priestley (1928), who maintained that the dermatogen of the stem apex of flowering plants grows rapidly in the tangential direction and becomes compressed into folds—the leaf-primordia—by its own growth, M. and R. Snow have demonstrated that when apices are incised vertically or transversely, the cuts gape. In other words, the superficial tissues are under tensile stress, not compressive stress.

The importance of obtaining experimental evidence bearing on the above hypotheses needs no emphasis. The formation of organs and tissues at the shoot apex depends on a whole nexus of factors including genetical, metabolic, and physical factors, while spatial arrangements are also involved. The action of particular factors must therefore be investigated for a better understanding of the complex situation at the growing-point. In the following sections an account is given of the methods used to test the hypotheses set out above and the results obtained.

#### MATERIALS AND METHODS

The shoot apices of large plants of *Dryopteris aristata* Druce were exposed by removing in the first place all the old leaves, and also the rolled leaves and larger primordia which surround the apex. The distal 2 cm. of shoot was then placed, apex upwards, under a binocular microscope and the scales removed by means of fine forceps, needles, and a fine brush. The apical cone, though covered in by scales, is itself devoid of scales. These grow out at the base of the cone and form a dense investment round the leaf-primordia. It is therefore rather difficult to remove all the scales without damaging some of the young primordia. Nevertheless, in order that the very youngest primordia may be seen it is essential that practically all the scales be removed. This whole apical region, which is of a medium-soft consistency, is very readily injured, particularly the young primordia. From small abrasions and punctures there is a copious exudation of fluid. Damaged tissues exposed to air quickly become brown.

The *apical cone* consists of a small protuberance on the rounded distal region of the shoot. The youngest primordia are formed on the apical cone; the older primordia and young rolled leaves are situated on the rounded *sub-*

*apical region.* The *apical meristem*, as defined by Wardlaw (1943)—a superficial layer of prism-shaped cells of distinctive appearance and chemical reaction—occupies the apex of the cone and extends for a variable distance down its flanks, but does not extend to the base of the cone.

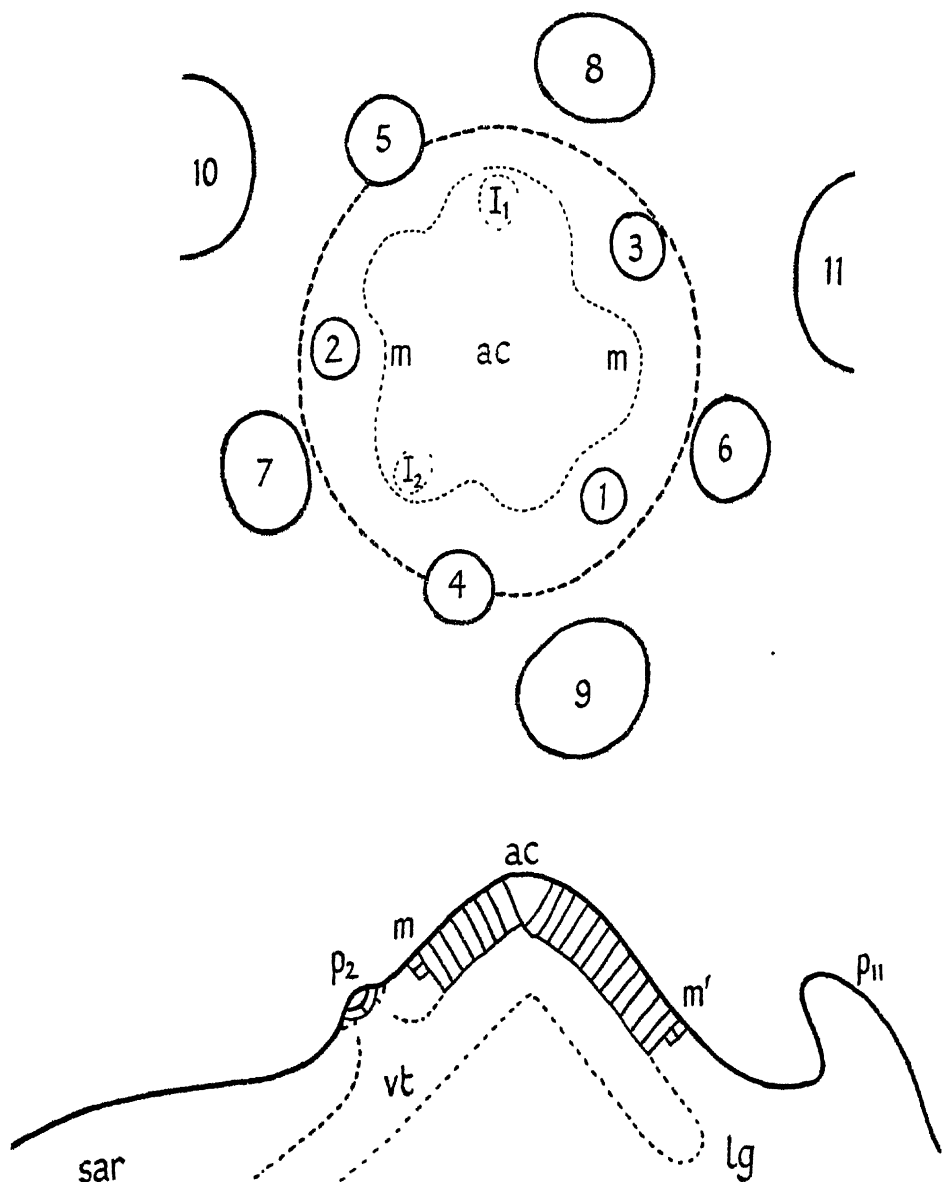
If a beam of light is directed on to an apex which has been prepared as described above, it can be seen that the three or four youngest leaf-primordia occupy positions a little above the base of the cone. The next three progressively older primordia are situated round the base of the cone, while still older primordia are in the region of transition between the base of the cone and the sub-apical region, or they may be entirely outside the cone (Text-fig. 1, Pl. III, Fig. 1). The primordia are arranged in a left- or right-handed spiral, usually with a fairly constant angle of divergence of  $138\frac{1}{2}^{\circ}$ . The very youngest primordium that can be observed under the binocular microscope at a magnification of 40 times as a very slight protuberance is indicated here as *primordium 1* ( $P_1$ ); the next older one is *primordium 2* ( $P_2$ ), and so on.

Following the terminology of M. and R. Snow (1931), the position of the next primordium—as yet invisible—to be formed is denoted by  $I_1$ , and the next after that by  $I_2$ . The position of  $I_1$  is not difficult to observe; it lies between and above *primordia 3* and *5* on a flat, or smooth, unoccupied flank of the cone. With suitable illumination even a small protuberance, were it present in this position, would be detected. Emergent bud-primordia have never been observed in uninjured, normal apices.

Two sets of experiments of a preliminary and exploratory character have been carried out.

In the first series an attempt was made to observe the distribution of growth at the apex. Apices from which all scales had been removed were treated with a suspension of lamp-black in a 10 per cent. aqueous solution of gum-arabic. By this means a thin and fairly uniform layer of lamp-black was deposited over the apical region. A suspension of lamp-black in water was not effective. Other finely divided materials, e.g. rice starch, were also tried, but the lamp-black gum-arabic suspension has proved most suitable. The treated apices were placed in moist peat and kept in a cool greenhouse. In some specimens there was evidence of growth in the course of a week (in August); all showed considerable growth in the course of 3 weeks. Where considerable growth had taken place, pale green tissue appeared through the black deposit; where some growth had taken place the deposit became thin and dispersed; where growth was slight or absent dense residues of lamp-black remained. At the end of 3 weeks a considerable number of scales had to be picked off to facilitate observation.

In the second series of experiments attempts were made to obtain actual demonstrations of the existence of tensile stresses in different regions of the apical cone. To this end superficial longitudinal incisions were made by means of a fine cataract knife in the axils of the primordia, or through both primordium and axil. Thus the effect of incising  $I_1$  and  $P_1$ , or  $I_1$  and  $P_2$ , or  $I_1$ ,  $P_2$ , and  $P_3$  could be compared, and so on. Another method consisted in



TEXT-FIG. 1. *Dryopteris aristata*: the apical cone as seen from above and in medium vertical section. The interrupted line separates the base of the cone from the sub-apical region (stippled). Leaf-primordia, 1, 2, 3, &c.;  $I_1$ ,  $I_2$ , positions of next two primordia to be formed. ac, apical cell. The apical meristem (m-m') does not cover the whole surface of the apical cone but extends a variable distance down its sides. vt, incipient vascular tissue below apex. sar, sub-apical region. lg, leaf-gap. (Semi-diagrammatic,  $\times 40$ .)

puncturing the leaf axils or the position of  $I_1$  by means of a very finely tapered sharp needle. In some instances  $I_1$  was the first position to be incised or punctured. Photographic records were made of a number of the specimens.

#### OBSERVATIONS ON THE DISTRIBUTION OF GROWTH

As further studies of growth by means of the lamp-black technique are in progress, the data so far obtained will be treated summarily. The earliest evidence of growth consisted in the emergence of scales. This took place during the first week and was followed, during the second and third weeks, by the growth of the older leaf-primordia at the base of the apical cone. These appeared as shiny, pale green mounds emerging from the black layer. The black deposit remained dense and undispersed in and above the axil of each primordium, indicating these as regions in which little growth had taken place. Where an actively growing tissue is immediately adjacent to, and in organic continuity with, a slowly growing tissue, the latter must be subjected to tensile stress. The observations so far obtained thus support the view that the superficial tissues of the apical cone on the adaxial side of developing primordia are subjected to tensile stress.

In the more actively growing specimens, evidence of growth over almost the whole apex could be observed, the original uniform black deposit becoming thin and dispersed to a mosaic-like pattern. Relatively dense deposits remained in and above the axils of all primordia and in the lateral spaces between primordia—i.e. the positions in which lateral buds subsequently develop (Wardlaw, 1943, 1943a). In a number of specimens, relatively undispersed areas of black deposit could be observed in two particular positions, those of the next two primordia to be formed, i.e.  $I_1$  and  $I_2$ . Such observations indicate that new primordia arise in positions of minimal growth. Lastly, it may be noted that the dispersion of the deposit increased progressively from the summit of the cone, downwards, i.e. growth is slowest in the region of the apical cell.

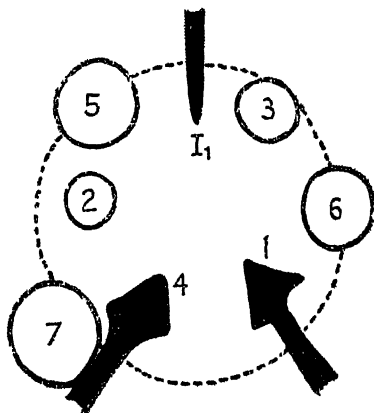
In some specimens the extreme apex of the cone had been injured. The black deposit was still present on the damaged necrotic region, but had become dispersed in the tissue immediately adjacent. This is what one would expect from a knowledge of the anatomy of damaged apices: in them the meristematic cells adjacent to the disrupted apical cell become parenchymatous and a considerable mass of tissue is formed.

#### OBSERVATIONS ON INCISED AND PUNCTURED APICES

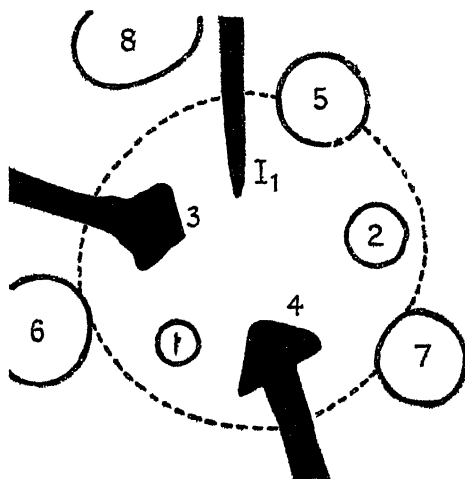
When the position of  $I_1$  was incised longitudinally, the cut neither opened nor closed, indicating the absence of any considerable tensile or compressive stress. When  $P_1$ ,  $P_2$ ,  $P_3$ , or  $P_4$  was incised so that the cut passed through the primordium and its axillary region, the cut usually opened up to form a wedge-shaped fissure, directed towards the shoot apex. In the sub-apical region the cuts neither opened farther nor closed.

In some instances the incisions through  $P_1$ – $P_4$  assumed the appearance of an arrow-head (Text-figs. 2, 3; Pl. III, Figs. 2, 3). In interpreting these

arrow-headed fissures caution is necessary. The apical meristem of *Dryopteris* is covered by a tough layer of cuticle, which offers considerable resistance to



TEXT-FIG. 2. Apex, with primordia on a right-handed spiral, incised at the positions of  $I_1$ ,  $P_1$ , and  $P_4$  as seen from above. In the two latter positions arrow-headed fissures have developed. ( $\times 30$ .) (See Pl. III, Fig. 2.)



TEXT-FIG. 3. Apex, with primordia on a left-handed spiral, incised at the positions  $I_1$ ,  $P_3$ , and  $P_4$ , as seen from above. ( $\times 30$ .) (See Pl. III, Fig. 3.)

the penetration even of sharp instruments. A finely drawn glass needle, for example, will bend and may even break before it will penetrate the apical cell. Furthermore, the tissues of the apical cone are of a watery consistency. Thus, when the cutting edge of a scalpel is thrust directly downwards on to the surface of the cone, it tends to plunge into the soft inner tissues once the resistance offered by the cuticle has been overcome. In so doing it may

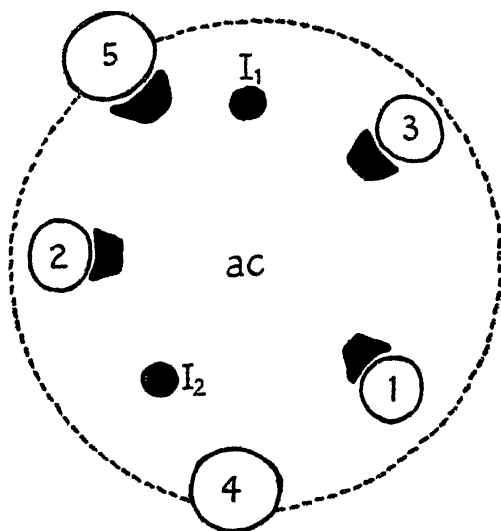
produce a triangular fissure. If the incision is made in the axil of a leaf, this initial triangular fissure can be seen to open out and the characteristic arrow-head fissure results. This takes place within a few minutes of the operation and can be observed under the microscope. On the other hand, triangular fissures in positions  $I_1$  and  $I_2$  do not open out. In a number of instances where the incisions had been made rather close to the apex, evidence that the tension was transmitted to that region was afforded by the appearance of brown colour in the cells lying between the apex of the cut and the apical cell (Pl. III, Figs. 5, 6). This colour is indicative of death in meristematic cells. Where two incisions had been made diametrically opposite it was not uncommon to find a brown necrotic line joining the two incisions, indicating that the stress had been experienced right over the summit of the cone.

By sliding the knife with a cutting action through a primordium *towards* the apex it was usually possible to avoid the bursting or tearing effect (with concomitant arrow-head wound) produced by a downward cut from the apical cone *towards* the primordium. These incisions also gaped in the case of  $P_1$ - $P_4$ , but did not open farther or close in the case of  $I_1$  or in interfoliar positions. Small incisions in the axils of  $P_1$ - $P_4$  also enlarged and gaped; in practice this operation so far has proved rather difficult to carry out satisfactorily.

In all, in this series of experiments, some twenty apices have been incised as described above. With occasional exceptions results of a high degree of consistency have been obtained. The position of the next leaf-primordium to be formed ( $I_1$ ), which is situated, as various observers have indicated, on the largest unoccupied flank of the apical cone, has consistently shown least response to incision, i.e. it is a region of minimal tensile stress. Although these preliminary results, based on a rather crude operational technique, should be accepted with caution, indications have been obtained that the youngest primordium  $P_1$  sets up less stress than  $P_2$ , and  $P_2$  less than  $P_3$  (Pl. III, Figs. 4-6).  $P_5$  and  $P_6$  also set up less stress in the apical cone than  $P_3$  when they lie outside the base of the cone. In fact, the data suggest that the tension induced by primordia of different ages *situated on the apical cone* is proportional to their size and to the size of their respective leaf-gaps; but in the case of older primordia outside of the cone, although their leaf-gaps are larger, and indeed continue to enlarge, their effect on the apical meristem diminishes progressively with their distance from it.

During the course of the experiments described above it became evident that what was really required to demonstrate tensile stress in different regions of the apical cone were small uniform punctures made in the axils of  $P_1$ - $P_5$ ,  $I_1$ , &c. This was effected by means of a needle ground to a very fine tapering point. This method has yielded conclusive results (Text-fig. 4, Pl. III, Figs. 7, 8, 9). The small circular holes made in positions  $I_1$  and  $I_2$  remained circular, or showed only a slight tangential distension, whereas those made in the axils of  $P_1$ - $P_5$  almost immediately became distended tangentially, the shape of the cavity being typically that of a truncated triangle or trapezium. The larger the primordium, the greater was the tangential distension. Punctures made

in the cone well above the leaf axil, i.e. close to the summit, did not become distended. When the apical cell was punctured there was a copious exudation of watery fluid which dried to a granular scab, but the hole did not enlarge. Punctures in those interfoliar positions at which lateral buds may develop remained unaltered, but others in positions close to the leaf-base became distended. When apices which had been placed in a 0.9 molar



TEXT-FIG. 4. Apex, with primordia on a right-handed spiral, punctured at positions  $I_1$ ,  $I_2$ ,  $P_1$ ,  $P_2$ ,  $P_3$ , and  $P_4$  as seen from above. At positions  $I_1$  and  $I_2$  the punctures (black) are approximately circular; in the leaf axils they become distended tangentially. *ac*, position of apical cell; stippling indicates the sub-apical region. This diagrammatic illustration serves as a key to Pl. III, Figs. 7, 8. ( $\times 40$ .)

solution of cane sugar for periods up to one hour were punctured the results were as already described, i.e. axillary punctures became distended. It might have been anticipated that by plasmolysing the tissues of primordia there would have been a marked diminution in the tensile stress in their axillary regions, but this result was not realized. The tissue at the base of the cone appeared to be more flaccid after treatment with the sugar solution, but there was no evidence of wrinkling or collapse. The tip of the apical cone appeared to be quite unaffected. This aspect requires further investigation.

Lastly it may be noted that where the apical cell had not been damaged new primordia were formed. In the course of 3 weeks the cuts became covered with a brown cork-like layer and were greatly enlarged tangentially.

#### DISCUSSION

Anatomical investigations had led the writer to the view that as fern leaf-primordia develop they induce tensile stress in the adjacent slowly growing

meristematic tissue of the apical cone. From these studies it had also been ascertained that the whole surface of the apical cone is not occupied by the distinctive prism-shaped cells of the apical meristem: these extend from the apical cell for a variable distance down the flanks of the cone, the lower regions being occupied by developing leaf-primordia and by parenchymatous tissue above the leaf axils. In certain interfoliar positions minute areas of meristematic cells may persist, these constituting the detached meristems from which lateral buds may arise subsequently (Wardlaw, 1943, 1943*a*). Broadly speaking, it appeared that the persistence of meristematic tissue in the lower part of the apical cone, and its capacity for giving rise to new leaf-primordia depend, among other factors, on the distribution of tensile stresses set up by the existing developing primordia. The largest vacant space in the basal region of the apical cone, on which the next primordium will arise, is, apparently, also the region of minimal tensile stress. These several ideas have now been tested experimentally. The data obtained support the views: (a) that developing leaf-primordia do induce tangential tensile stress in the apical cone; and (b), that each new leaf-primordium is situated in that region of the apical meristem in which tensile stress is minimal.

As noted in the Introduction, Hofmeister advanced a mechanical explanation of phyllotaxis based on the setting up of tensions at the growing-point in relation to the outgrowth of lateral members. He has suggested: (1) that the outer walls of the superficial cells of the shoot apex offer resistance to the lateral outgrowth of new organs (primordia); (2) that this resistance is not uniform over the growing-point, new lateral organs appearing in regions of greatest elasticity; (3) that in proximity to the last-formed primordia the superficial membrane, being already stretched, has minimal elasticity; (4) that a new primordium will appear in that position on the apex which lies farthest from the last-formed primordia, i.e. in the position of maximal elasticity and minimal tension. While Hofmeister's observation that new leaf-primordia arise in the largest gap has been accepted by many investigators of phyllotaxis, his mechanical theory outlined above has received little support.

Whether there are differences in the elasticity of the outer membrane in different regions of the growing-point, or whether the outgrowth of primordia is to be explained in terms of factors of a different kind, e.g. factors of growth, cannot be decided on the evidence at present available. A fuller discussion of Hofmeister's theory will therefore be deferred until later.

According to M. and R. SNOW (1931, 1933, 1935, 1947, 1947*a*) each new leaf is formed 'in the first available space on the apex above and between the existing leaves or other contact members in the top cycle—that is to say, in the first space which attains both some necessary width and some necessary distance below the extreme growing point' (1947). The present writer is in agreement with this description of the position of the new primordium and has indicated a physical factor which, together with metabolic and other factors yet to be considered, may be held responsible for determining the formation of leaves and their arrangement on the shoot axis.

M. and R. SNOW (1947) have shown that when the apices of various flowering plants were incised either in the vertical or radial plane in the presumptive areas of the next leaves to be formed, the cuts typically gaped, i.e. the meristematic tissue was under tensile stress in all directions. They also observed that wounds on the abaxial side of young primordia did not gape or did so very slightly. While they have used these experimental data to refute the view that leaf-primordia arise as a result of compressive stress in the superficial tissues of the apex, they have not used them to elaborate the view that tensile stress in the apical meristem may be an important factor in determining leaf position. Their data, however, support the first hypothesis advanced here. Earlier investigators of phyllotaxis, e.g. Schwendener and others, entertained the view that the contact pressure between rapidly developing primordia was a factor which determined the positions of new primordia. Church (1904) has dealt very thoroughly with this and related hypotheses and has shown that while there is ample evidence of contact pressure among the older primordia—where mutual compression may be a real and indeed evident factor—primordia at the time of their inception are widely spaced and quite separate from one another. In this connexion the apices of *Dryopteris*, *Nymphaea*, &c., are particularly mentioned and illustrated by Church.

M. and R. SNOW (1931, 1947) have shown that if either of the two primordia adjacent to the next primordium to be formed is incised, the new primordium is always displaced towards the wound. Thus, in Text-fig. 1, if  $P_2$  were cut the anticipation would be that  $I_2$  would be displaced towards it. But this is just what one would expect on the hypothesis now advanced: for if  $P_2$  is injured at a very early stage its further development will cease, and the tangential stress which it will induce will be slight. But tension will continue to be induced by the continued development of  $P_1$  and  $P_4$ . The region of minimal stress will therefore now lie nearer to  $P_2$  than in an untreated apex. Experiments to test this inference are in progress.

As noted in the Introduction, a fuller consideration of phyllotaxis in ferns is being reserved for a later paper, in which a comprehensive anatomical account of the formation of leaf-primordia in *Dryopteris* will be given, but some points of immediate relevance will be discussed here.

Since the rate of growth of the short apex in *Dryopteris* is slow and the enlargement of the adjacent sub-apical region rapid, it might perhaps be thought that the whole apical cone, and more particularly its basal region, would be subject to tangential tensile stress. If this is so, the magnitude of the stress is not great, since longitudinal cuts in interfoliar positions at the base of the cone do not open out. A consideration of the volume relationships of the apical cone and of the adjacent sub-apical region shows that the latter would have to grow very rapidly indeed before the former was subjected to tensile stress.

From the time of its inception the growth of a leaf-primordium is rapid relative to that of the tissue in and above its axil; indeed the growth of

tissue in the leaf axil may actually be inhibited, *Matteuccia struthiopteris* affording an extreme example of this phenomenon (Mekel, 1933). Growth is particularly active in the leaf-base, this being largely due to the development of parenchyma in the cortex and pith.

If the width of the leaf-gap affords a measure of the tangential stress exercised by the enlarging pith of the leaf-base on the incipient vascular cylinder of the shoot, it may be supposed that the leaf-base as a whole subjects a still more extensive area of the superficial apical meristem to tensile stress. There is support for the view that when a region of the apical meristem is subjected to tensile stress its constituent cells divide repeatedly by periclinal and anticlinal walls and give rise to epidermis and cortical parenchyma. In point of fact a band of cortical parenchyma, narrowing progressively in the acropetal direction, extends upwards from the axil of each primordium. Just at or above the level where the leaf-gap 'closes', i.e. where two meristeles appear to become conjoined, the stress exerted by the leaf-primordium becomes minimal, or disappears, and in this localized region a small group of superficial meristematic cells may persist. It is from these groups of cells, and from them alone, that lateral buds develop. In *Dryopteris* these residual cells of the apical meristem are difficult to distinguish but in *Matteuccia* and *Onoclea* they can readily be observed (Wardlaw, 1943, 1943a). There are many observations which suggest that when cells of the apical meristem are transformed into parenchyma the incidence of tensile stress is one of the factors at work.

As noted above, the distinctive prism-shaped cells of the apical meristem, from which leaf-primordia and buds originate, do not extend uniformly downwards over the whole surface of the apical cone. They stop short some distance above the leaf-axil, according to the size of the primordium, but extend farther down the sides of the cone in the interfoliar positions. In particular, the apical meristem extends far down the cone on the flank between the third and fifth primordia (Text-fig. 1). It has now been shown experimentally that this is the region where tensile stress is minimal: it is also the presumptive position of the next primordium to be formed.

Buds and leaves originate from comparable cells of the apical meristem: in each instance there is evidence that the new organ is situated in a region of minimal tensile stress. In other words, in the absence of tensile stress, meristematic cells are able to persist and function as meristematic cells; for example, they are capable of giving rise to new organs. On the other hand, when actively growing meristematic cells are subjected to tensile stress they develop into parenchymatous tissue. Why this should be is still obscure. The problem is clearly one of very great interest and importance and should receive attention at the hands of the physiologist or biophysicist.

Lastly, it may be noted that the incidence of tensile stress depends on the distribution of growth. Hence the fundamental problem underlying that which has been discussed here relates to the factors which control or determine the different rates of growth in different regions of the shoot apex.

## SUMMARY

The hypothesis is advanced that as each leaf-primordium develops at the growing-point of *Dryopteris aristata*, it induces a tangential tensile stress in the apical meristem above its axil. A further hypothesis is that the next primordium to be formed arises in that region of the apical cone in which tensile stress is minimal.

Preliminary studies of the distribution of growth in specimens in which the apical region had been covered with a thin layer of lamp-black support these views.

When apices were laid bare and incised and punctured by techniques which are described, positive evidence was obtained of the existence of a region of tensile stress in and above the axil of each primordium. But stress was slight or absent in those regions in which the new primordia would arise. The largest unoccupied space on the apical cone, above the level of the last-formed primordium (which as other investigators have pointed out is the position in which the next leaf-primordium will arise), is also the region in which tensile stress is minimal.

The absence of tensile stress is thus in some way, as yet obscure, related to the organ-forming activity of the apical meristem.

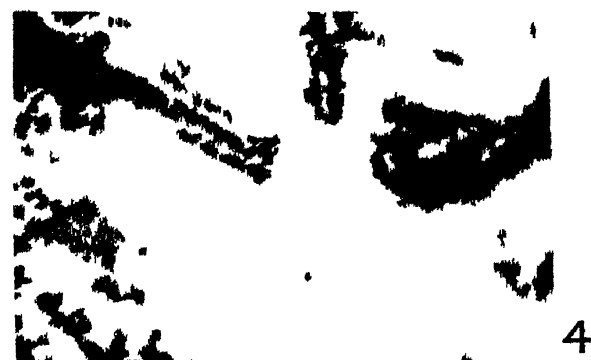
A full consideration of the data which have been obtained is being reserved until later, but a number of points of immediate relevance are discussed.

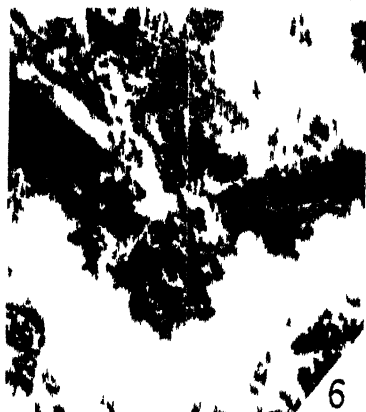
The writer has pleasure in acknowledging his indebtedness to Mr. E. Ashby for the photographic illustrations.

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# EXPLANATION OF PLATE

Illustrating Professor C. W. Wardlaw's article, Experimental and Analytical Studies of Pteridophytes, XI.

The figures are from untouched photographs by E. Ashby.

*Dryopteris aristata* (all figures are views of the shoot apex from above,  $\times 40$ ).

Fig. 1. Large apical cone with leaf-primordia.

Fig. 2. The positions of the next leaf-primordium to be formed (top) and of primordia 1 and 4 have been incised (see Text-fig. 2).

Fig. 3. The positions of  $I_1$  (top),  $P_3$ , and  $P_1$  have been incised (see Text-fig. 3).

Fig. 4. The positions of  $I_1$ ,  $P_3$ , and  $P_2$  have been incised.

Fig. 5. The positions of  $P_1$  (left) and  $P_2$  have been incised.

Fig. 6. The positions of  $P_2$  (right) and  $P_3$  have been incised.

In Figs. 5 and 6 the region of the apical cell has been injured as a result of the transmission of the stress from the incisions.

Figs. 7 and 8. Axils of primordia  $P_1$ ,  $P_2$ ,  $P_3$ ,  $P_4$ , (right-handed spiral) punctured. The holes have become distended tangentially. Those in the positions of  $I_1$  (top centre) and  $I_2$  (bottom centre approximately) have remained circular (see Text-fig. 4).

Fig. 9. Axils of primordia  $P_3$  (top right),  $P_4$  (top left),  $I_1$  (top centre), and  $P_1$  (bottom right) punctured.  $I_1$  has remained approximately circular; the others have become distended tangentially. (Primordia in right-handed spiral.)



# Studies in the Physiology and Morphology of *Penicillium notatum*

## II. Production of Penicillin by Mature Hyphae

BY

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With nineteen Figures in the text

### INTRODUCTION

IN an earlier paper (Whinfield, 1947) the question was raised as to which regions of the hyphae of *Penicillium notatum* are concerned in penicillin production. The presence of penicillin in cultures of young germ-tubes of average length  $30\mu$  was reported. Data relating to the further question of penicillin production by older regions of the hyphae are discussed in the present paper. This investigation has entailed observations of the course of penicillin production and of morphological changes in comparable cultures.

### MATERIALS AND METHODS

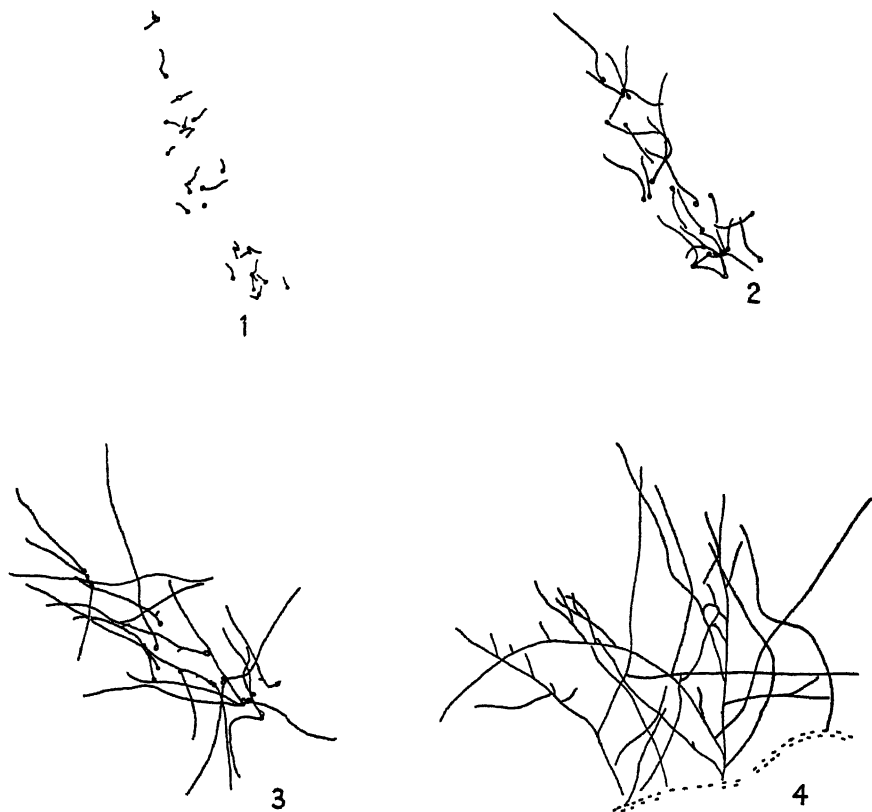
Because of the density of cultures necessary to produce quantities of penicillin detectable by ordinary assay methods, direct microscopic observation of the condition of hyphae was impracticable. Moreover, techniques for assaying the minute amounts of penicillin produced by small numbers of conidia were insufficiently accurate. Hence comparatively dense cultures were used and the rate of growth studied by means of fresh- and dry-weight determinations, while relevant morphological data were obtained from cultures grown from a few conidia.

Conidia were harvested from stock cultures and prepared for use as previously described. From a concentrated conidial suspension (C) of washed conidia three further suspensions (C/10, C/100, and C/1,000) were prepared by making appropriate successive dilutions. These four suspensions were used as inocula for four series of cultures. The culture technique was that previously used for demonstrating production from young germ-tubes, i.e. 7.5 c.c. of liquid corn-steep medium were held in glass wool in a Petri dish and 0.5 c.c. of the inoculum was spread over the surface of a disc of permeable cellophane resting on this glass wool. The four series of cultures were then incubated simultaneously at 23–4° C. The corn-steep medium was one used in commercial penicillin production. The industrial strain of *P. notatum* known as N.R.R.L. 1249 B. 21 was used throughout.

Two experiments were carried out as follows:

*First experiment*

Every day for 5 days a sample of three dishes was taken from each series. The medium from all three was drawn off, mixed, and assayed by the Oxford



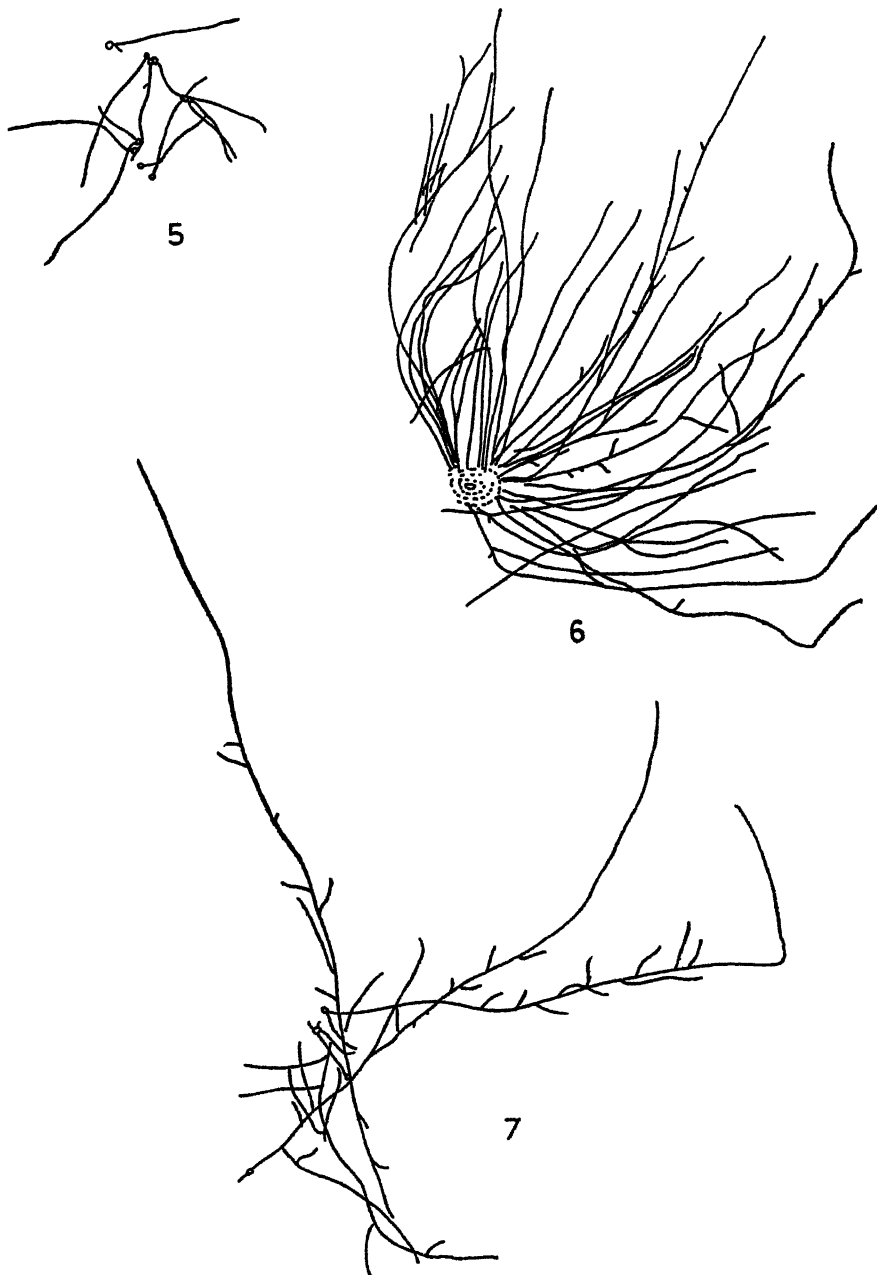
FIGS. 1-4. Stages in the development of *P. notatum* from conidia: (1) 13 hours, (2) 18 hours, (3) 22 hours, (4) 36 hours after inoculation into corn-steep medium.

cup method. The fungus was scraped off the cellophane and its fresh and dry weights determined, the weights of the three duplicates being averaged.

*Second experiment*

Every other day for 6 days a sample of three dishes was removed from each series and treated as in the first experiment. At the same time the medium of all remaining cultures (i.e. those which were to form subsequent samples) was changed by transferring the cellophane bearing the fungus to a fresh dish of medium.

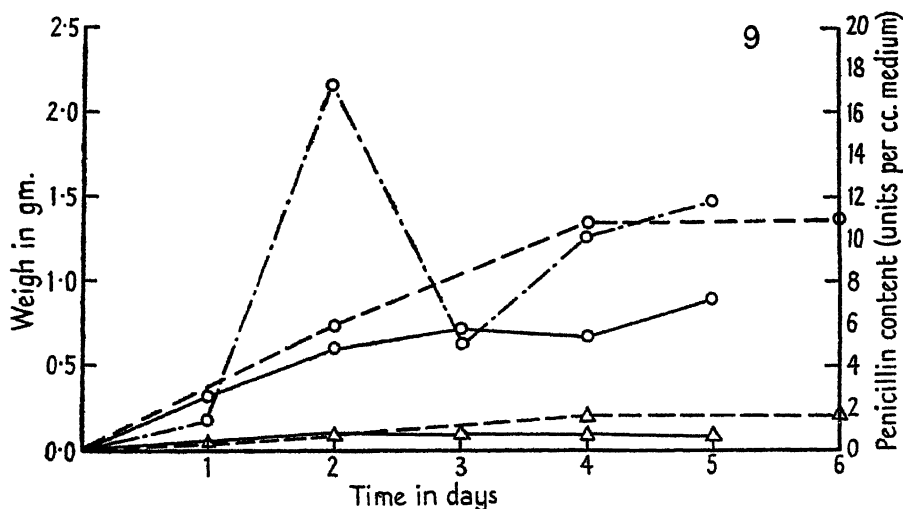
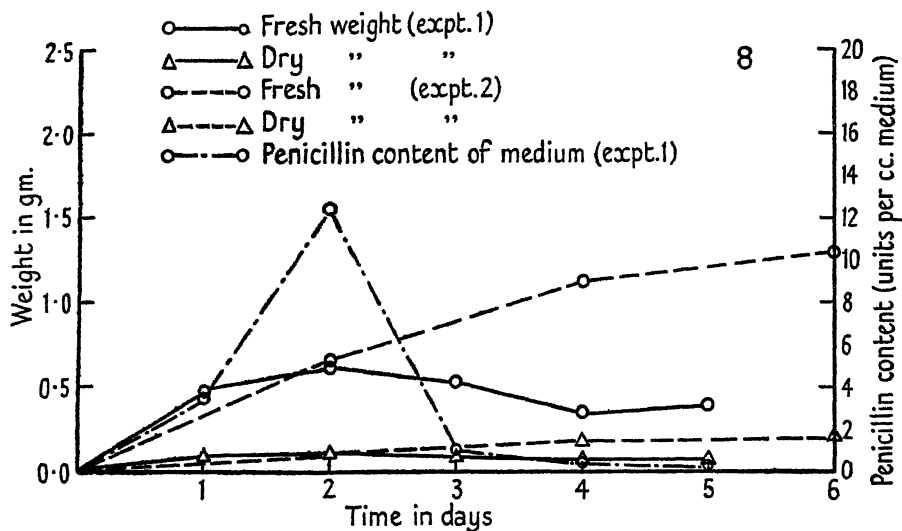
After 6 days the cellophane became so rotted that the fungus could not readily be separated from it; hence these experiments were not prolonged.



FIGS. 5-7. The effect of conidial density on development: (5 and 6) colonies 22 hours after inoculation; (7) colony 28 hours after inoculation.

*Morphological observations*

Streaks of corn-steep medium containing 0.5 per cent. agar on cover-slips were inoculated at one end with a small number of conidia. These cultures



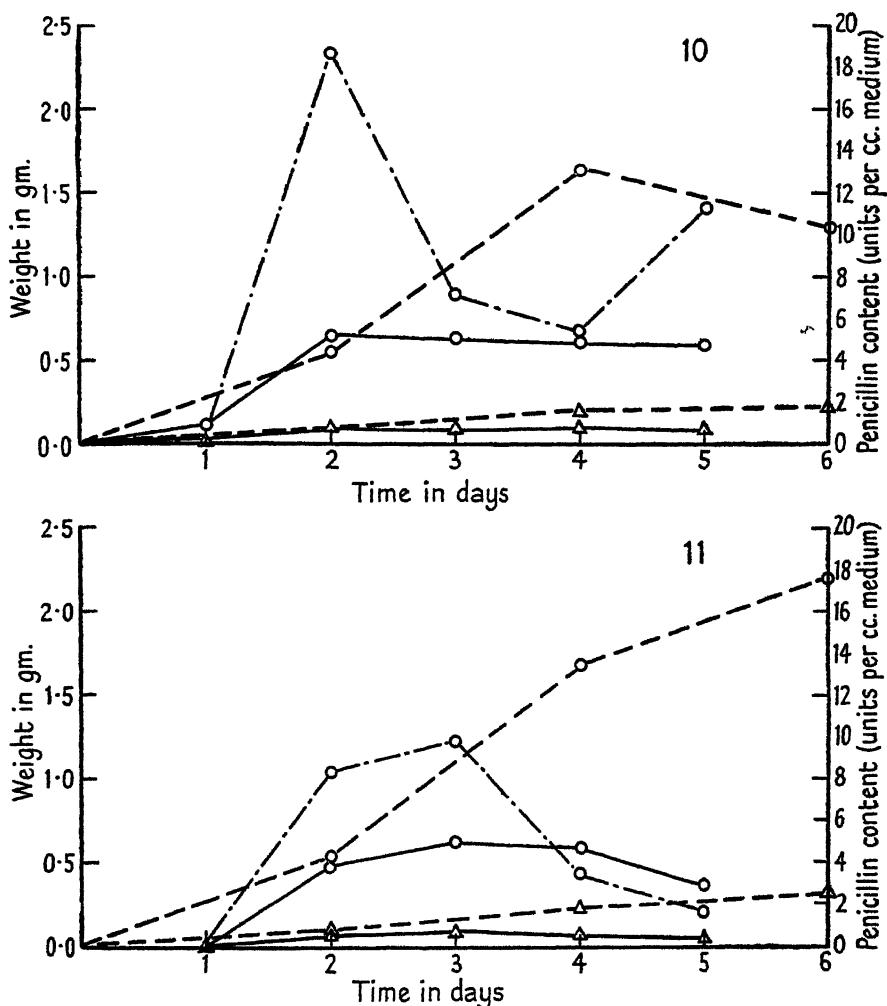
FIGS. 8-9.

FIGS. 8-11. Change with time in fresh and dry weights of *P. notatum* colonies (experiments 1 and 2) and penicillin content of the medium (experiment 1): (8) C cultures; (9) C/10 cultures; (10) C/100 cultures; (11) C/1,000 cultures.

were incubated at 23-4° C. and the course of their development followed by means of camera-lucida drawings.

## OBSERVATIONS AND RESULTS

Figures 1-4 illustrate stages in the development of a small number of conidia in a streak of medium. Under these conditions the primary germ-tube

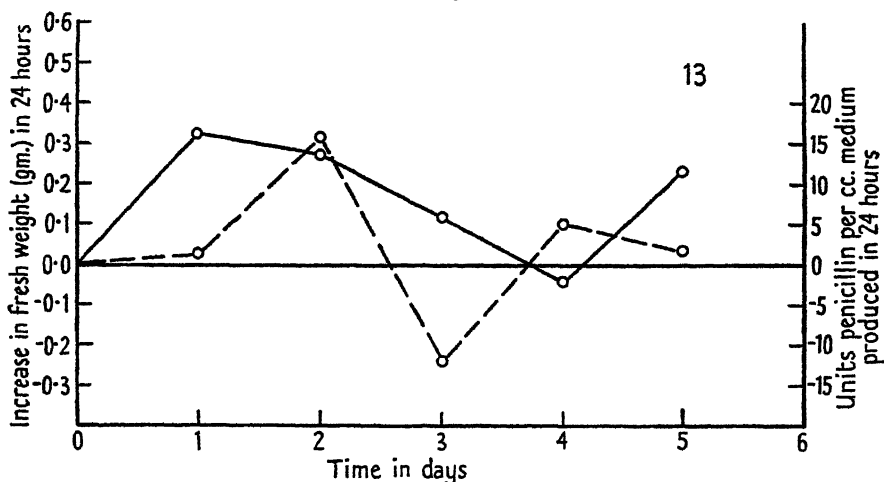
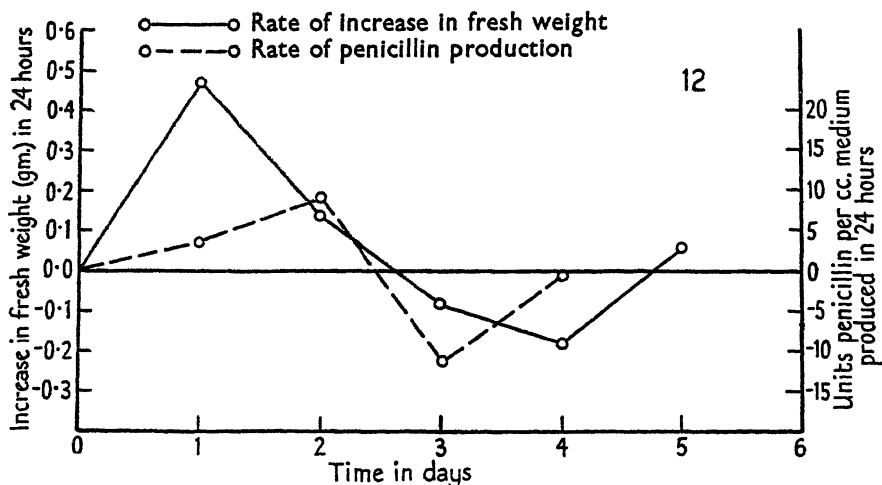


FIGS. 10-11.

appears about 9-10 hours after inoculation and elongates for about 10 hours before branching begins. Thereafter branching continues rapidly.

The elongation of the primary germ-tube was found to be much more rapid from conidia in a dense cluster than from relatively isolated ones, as illustrated by a comparison of the dense 22-hour culture in Fig. 6 with the sparser colonies in Figs. 5 and 7. That in Fig. 5 is the same age but the hyphae are much shorter. That in Fig. 7 is 6 hours older but the hyphae are only equal in length to those of Fig. 6.

In denser cultures, however, branching was found to be largely or completely inhibited. The hyphae in Fig. 7 show considerable branching, while the more densely packed ones in Fig. 6 show very little.

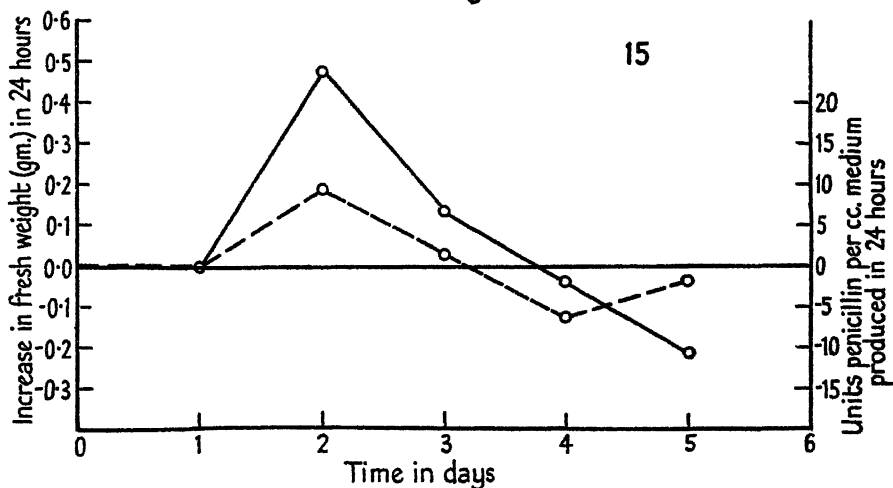
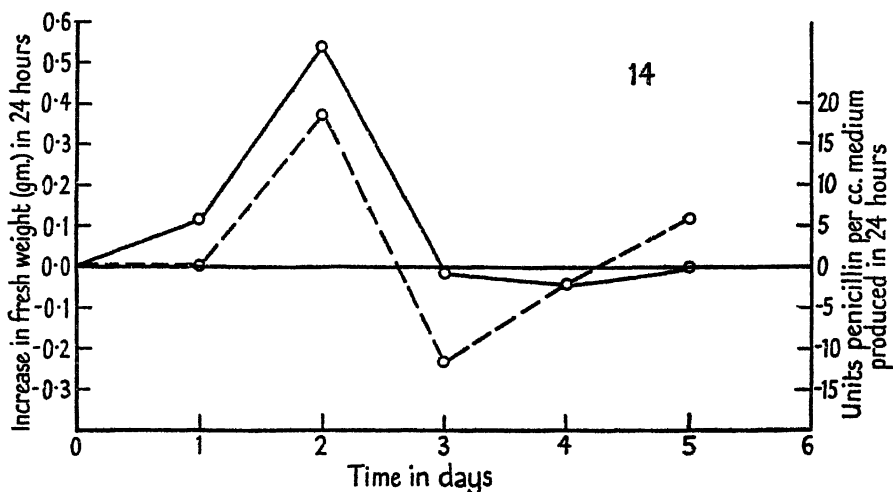


FIGS. 12-13.

FIGS. 12-15. Rate of growth (fresh weight) and of penicillin production in experiment 1: (12) C cultures; (13) C/10 cultures; (14) C/100 cultures; (15) C/1,000 cultures.

The data relating to the two experiments on growth and penicillin production are illustrated graphically in Figs. 8-19. In the first experiment the curves of mycelial weights are approximately sigmoid in form, the later phase being usually marked by some decrease due, presumably, to hyphal autolysis. In the second experiment changing the medium prolonged the period of active growth but did not essentially alter the form of the curves. In both experiments the maximum growth was attained during the first day by the C and

C/10 cultures but not until the second (first experiment) or third day (second experiment) by the C/100 and C/1,000 cultures.

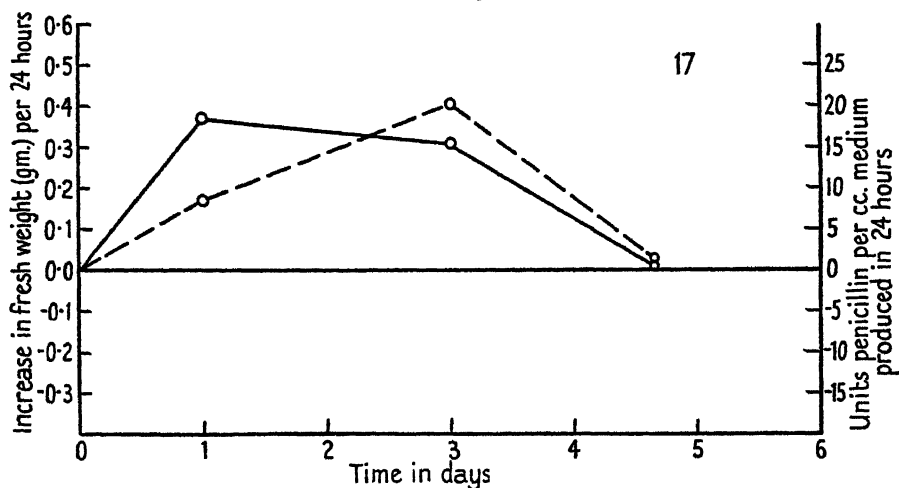
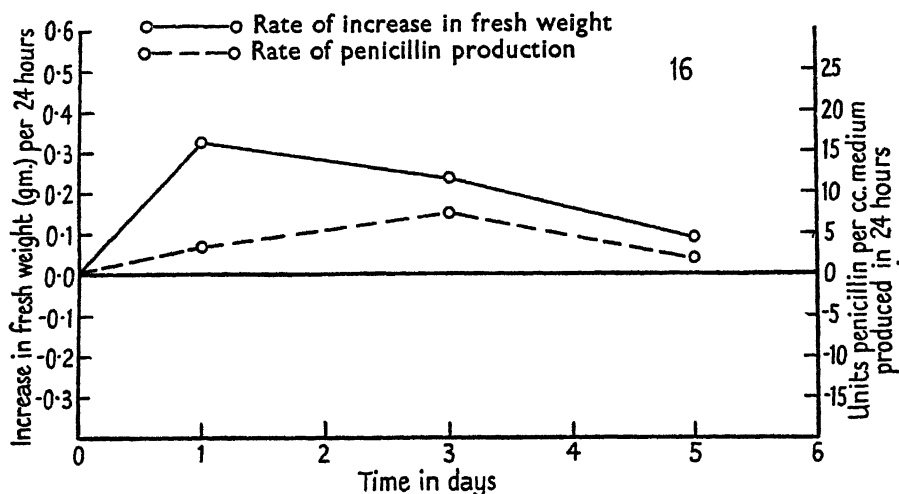


FIGS. 14-15.

The principal observations made may be summarized as follows:

- (i) that during the first 24 hours the primary germ-tube elongates more rapidly in dense clusters of conidia;
- (ii) that branching is inhibited in densely aggregated hyphae;
- (iii) that the maximum rate of growth (in terms of weight) is attained in 1 day in cultures grown from very dense inocula but not for 2 or 3 days in cultures grown from relatively dilute ones, and

- (iv) that the maximum rate of penicillin production is attained simultaneously in cultures grown from inocula of different density and coincides with the maximum growth rate in those grown from diluter inocula.



FIGS. 16-17.

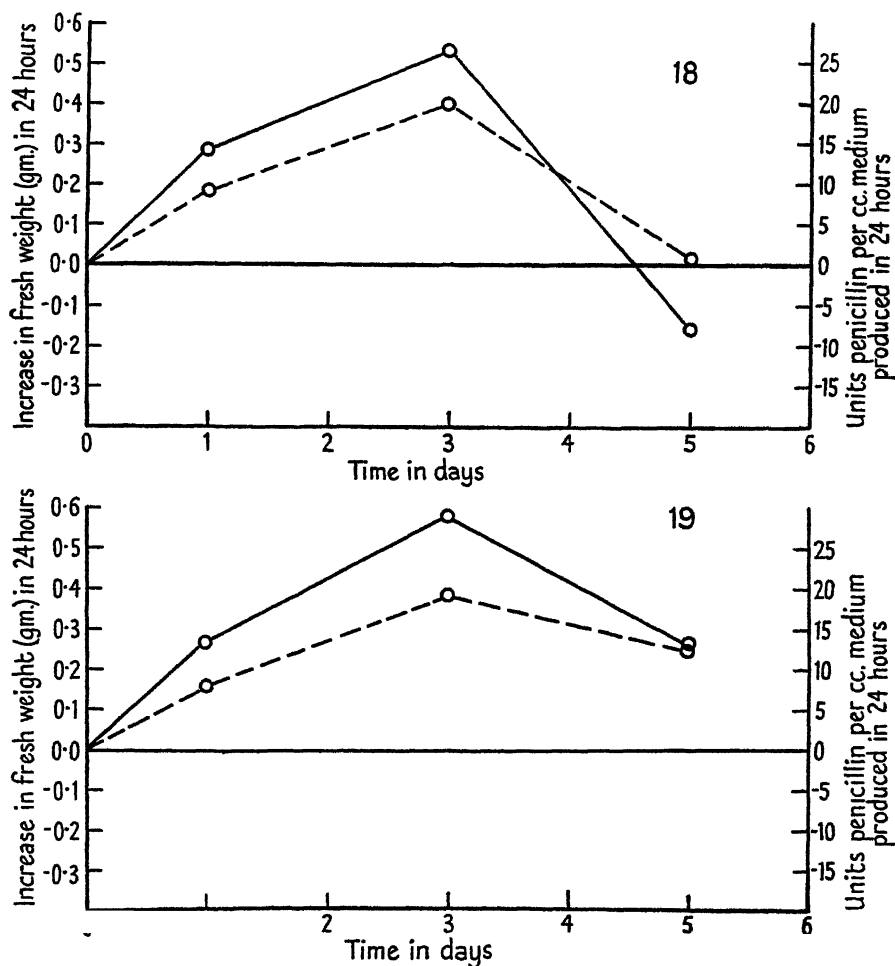
FIGS. 16-19. Rate of growth (fresh weight) and of penicillin production in experiment 2: (16) C cultures; (17) C/10 cultures; (18) C/100 cultures; (19) C/1,000 cultures.

### DISCUSSION

On the basis of the above observations the following hypothesis is advanced.

The density of the C and C/10 cultures results in a rapid growth of the germ-tubes during the first 24 hours and consequently in the accumulation of a sufficient concentration of staling substances in the medium to diminish the growth rate. Moreover, owing to the dense aggregation of the hyphae, branch-

ing is largely or completely inhibited. The growth of these cultures is thus due almost entirely to the growth of the primary germ-tubes and attains its maximum rate during the first 24 hours.



FIGS. 18-19.

The much lower growth-rate of the C/100 and C/1,000 cultures during the first 24 hours is due to the smaller number of growing-tips present and to the fact that the less crowded the germ-tubes the slower their growth. Therefore after 24 hours the concentration of staling substances in the medium is much lower than in the case of the denser cultures. Moreover, the cultures are sufficiently sparse after 24 hours for branching to occur, and this results in the increase in growth-rate shown during the second day. If staling substances are removed by changing the medium this increase continues for a third day.

If this hypothesis is correct, it becomes clear that maximum penicillin production is associated with the presence of that region of the hyphae in which branch tips may appear, but is independent of whether such tips are in fact formed. It appears that production starts just behind the growing-tip, reaches its maximum in the 2–3-day-old region (where branching may occur), and gives way to penicillin destruction in the older autolysing regions.

These conclusions agree with those of Pontecorvo (1945), who placed a colony of the fungus grown on cellophane on the surface of agar for a short time and then tested the inhibitory power of the agar from below different regions of the colony. He found the inhibition to be greatest from a region about 1 cm. behind the outer edge of the colony and to decrease progressively on passing either inwards or outwards from this region, and concluded that, in his strains, production appeared to be associated with already established hyphae and not to be connected with cell multiplication. No morphological examination was made of the colony, however, and therefore it might have been the case that the greatest density of hyphal tips occurred in this region and that the maximum penicillin production was associated with them.

The difficulty of using the same culture for measuring penicillin production and obtaining morphological data is the chief obstacle in the investigation of this problem. It must be emphasized that, although the present results provide evidence for the conclusions advanced, they do not represent proof because the morphological condition of the cultures in which production was measured was inferred from indirect observations.

#### SUMMARY

The production of penicillin by germ-tubes of *Penicillium notatum* 30  $\mu$  in length has already been demonstrated. This paper deals with the question of penicillin production by older regions of the hyphae.

The production of penicillin appears to reach its maximum rate in 2–3-day-old regions of the hyphae where, under suitable conditions, branching occurs. Production is not, however, directly associated with the formation of branch tips.

In the presence of still older mycelium, where autolysis is believed to occur, there is a rapid destruction of penicillin.

The writer wishes to express her gratitude to Professor C. W. Wardlaw for his supervision and constant help in the carrying out of this work, and for his valuable criticism in the preparation of this paper. She also wishes to acknowledge her indebtedness to Imperial Chemicals (Pharmaceuticals) Ltd. for a grant which made the work possible.

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# Aluminium in Plants and its Relation to Plant Pigments

BY

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AS a natural sequel to the study of the colour changes in the flowers of *Hydrangea macrophylla* (Chenery, 1937) an attempt was made to find other plants of like behaviour. This search has been carried out sporadically over the last 12 years, and a brief summary was published in 'Nature' (Chenery, 1946). It is the purpose of this paper to describe what may be termed 'Hydrangea-like' plants. The flowers of such plants, if pink, will change to blue when the plants are transferred to a more acid soil or after treatment of the soil with aluminium salts; if blue they will change to pink when grown in a lime-rich or less-acid soil.

## HISTORICAL

During the last 50 years several plant physiologists have endeavoured to effect flower colour changes by various chemical treatments. All were doubtless familiar with the influence of aluminium salts on *Hydrangea* flowers and naturally tried to produce similar changes in other plants. Thus, at the conclusion of his experiments on *Hydrangeas*, Molisch (1899) pointed out that all anthocyanins do not behave in the same way and that this might be one of the reasons why it had not been possible to change the colour of the flowers of other plants by soil treatment. However, the next year Miyoshi (1900) claimed that the flowers of *Callistephus chinensis* and *Campanula alliariifolia* could be changed from lilac to blue and flowers of *Lycoris radiata* from red to lilac by treatment with aluminium salts. He did not record the exact procedure nor whether the aluminium was absorbed through the roots or the cut stems. A large number of substances, including alum, were used by Kraemer (1906) in order to bring about colour changes in roses and carnations, but the results were never marked and were usually quite insignificant. Vouk (1908) unsuccessfully endeavoured to alter the colour of flowers of *Phlox decussata* by watering the plants with alum.

Using a Knopp's solution to which 0.01 per cent. aluminium nitrate was added, Kratzmann (1914) caused red cabbage to produce a blue anthocyanin, an effect which was never obtained in soil cultures. He also claimed that cut twigs of red beech and hazel produced a blue pigment when placed in aluminium solutions. Molisch (1922) recorded his failure to promote changes to blue in red *Primula sinensis*, red cyclamen, and red roses by alum applications. He concluded from this that *Hydrangea macrophylla* contained a characteristic

anthocyanin. Stocklasa (1922) also in the same year, experimenting with *Fuchsia* sp., *Matthiola annua*, *Papaver somnifera*, *Nicotiana longifolia*, and *Rhododendron indicum*, recorded that treatment with aluminium salts tended to intensify flower colour, and to convert white flowers to red and red ones to violet or blue. Stocklasa's coloured illustrations did not, however, bear out all his claims.

It is not surprising that the attempts to induce colour changes in flowers other than Hydrangeas gave negative or unconvincing results as the problem appears never to have been critically analysed. Tests on the effect of various salts on extracts of petal-colouring matters would have eliminated all the obviously unlikely plants, and observations of their soil-reaction preferences would have reduced their number still further. The likelihood of added substances being absorbed by the soil and thereby rendered non-available to the plant was also neglected.

Analytical investigations (Chenery, 1937) on Hydrangea plants revealed that aluminium and not iron was the element responsible for the change of flowers from pink to blue. Similar data were obtained by Storck (1942) and Allen (1943). The latter worker was unable to produce blue flowers in plants grown in acid culture solutions containing abundant iron but no aluminium, blue flowers being produced only when aluminium was present.

The exact mechanism of the Hydrangea flower colour change is still somewhat obscure. It has been ascribed to an organic colloidal dispersion effect by Robinson (1939) accompanied by a reduction in concentration of anthocyanin. Aluminium may possibly improve the dispersion of the colloidal pigments, but this has not been experimentally verified. Chenery (1937, 1946) ascribed the colour change to the formation of a blue-coloured acid-stable aluminium lake which may be regarded as a colloidal complex or a loose combination of the delphinidin pigment and aluminium. Since it has not yet been demonstrated that it is possible to reduce the anthocyanin concentration of pink Hydrangea flowers, and at the same time change their colour to blue, by any means other than supplying available aluminium, the second view has been adopted in this paper.

From the researches on 'Hydrangea-like' plants three conditions emerge that have to be fulfilled before any soil-induced flower colour change will take place. The plant must (1) have a delphinidin flower pigment which in an acid cell-sap is normally pink but is blue in the presence of excess aluminium; (2) be able to accumulate aluminium; this is a specific characteristic manifest only when sufficient is available, e.g. in acid soils; (3) have a wide range of reaction tolerance.

The logical approach to the search for 'Hydrangea-like' flowers would be, firstly, to examine as many plants as possible for the presence of delphinidin pigments and ascertain whether they will absorb aluminium. Secondly, to search the literature for records of blue-flowering plants that are natural aluminium-accumulators or are likely to be so. These may be defined as plants which absorb aluminium to the extent of 1,000 p.p.m. of dry matter.

Finally, when such a blue-flowered aluminium-accumulator is found it must be grown under aluminium-free conditions in order to determine whether its flowers will change to pink.

#### EXPERIMENTS WITH PLANT PIGMENTS

Since Potapov (1934) found that extracts of *Rhamnus* sp. berries gave a blue colour with small quantities of aluminium salts, it was considered in the present study very likely that other plant pigments would behave similarly.

The maceration experiments as performed on *Hydrangea* sepals afforded a very rapid means of testing with aluminium salts for anthocyanin colour changes. By this procedure it was ascertained that aluminium produced four types of colour change in plant-colouring matters, viz.: (1) mauve or red to pure sky blue; (2) purple to deep blue; (3) red to violet; (4) pale yellow to very deep yellow (anthoxanthins).

All the above effects were confirmed when the respective pigments, extracted with acetic acid and precipitated with ether, were tested with dilute aluminium sulphate solution. The anthoxanthin change was later found to have been recorded by Keegan (1899) and Perkin and Everest (1918). Shibata, Shibata, and Kasiwagi (1919) apparently produced violet colorations by treating certain flower petals with aluminium sulphate, but specific pigments were not mentioned.

While this investigation was in progress the survey of anthocyanins made by Robinson and Robinson (1931, 1932, 1933, 1934) was found to give striking confirmation of the utility of the aluminium test as a means of determining anthocyanin types. It appeared that pigments giving changes (1), (2), and (3) were produced by delphinidin, petunidin, and cyanidin glycosides respectively. The plants tested are listed below:

Sky-blue colorations were obtained from pink and red flowers of the following plants which contain delphinidin pigments: *Androsace* sp., *Deutzia scabra*\*, *Erica cinera*\*, *Erinus carmineus*\*, *Hydrangea macrophylla*, *Nicotiana sieberi*, *Nymphaea* sp., *Phlox amoena*\*, *Primula obconica*, *Pulmonaria* sp., *Verbena* sp., *Veronica* sp.

Sky-blue colorations were also obtained from the following purplish-blue, purple, or lilac-coloured flowers: *Callistephus hortensis*, *Campanula* 4 spp., *Capsicum chamaelon*\* (fruits), *Ceanothus* sp., *Cineraria* sp., *Convolvulus mauritanicus*, *Crocus* sp., *Delphinium* sp., *Erinus alpinus*, *Iris imperator*, *Lobelia cardinalis*, *Pentstemon heterophyllus*, *Rhamnus frangula* (berries), *Syringa vulgaris*, *Tulipa* sp., *Verbena radicans*, *Veronica* 2 spp., and *Viola odorata*. Deep blue colorations were obtained from purple flowers of *Petunia violacea* and the berries of *Ligustum vulgare* which contain the pigment petunidin.

Violet colorations were obtained from the pink or red flowers of the following plants which contain cyanidin pigments: *Bellis perennis*, *Brassica campestris*\* (roots), *Camellia japonica*, *Centaurea cyanus*, *Cotoneaster*

*microphylla*\* (fruit), *Crataegus oxyacanthoides* (fruit), *Dianthus* sp., *Epacris* sp., *Gaultheria procumbens* (fruit), *G. shallon* (fruit), *Grossularia* 2 spp. (fruit), *Hibiscus* 2 spp., *Leycesteria formosa*, *Lilium* 4 spp., *Lonicera* 2 spp.\* (fruit), *Potentilla willmottiae*, *Rheum rhaponticum* (stalks), *Rosa* 2 spp., *Rubus fruticosus* (fruit), *Weigelia* sp.

Those species marked with an asterisk had not been tested by previous workers. The aluminium test would appear to be more suitable for anthocyanins *in situ* than the iron test as there is no tannin interference.

#### FURTHER OBSERVATIONS ON HYDRANGEA PIGMENTS

Robinson and Robinson (1932) found cyanidin derivatives in the leaves of *Hydrangea* together with the cyanidin leuco-base. The stem spots and leaf-buds of *Hydrangea* are usually pigmented, especially in the coloured varieties; a colour change (cf. Allen, 1943) takes place in this pigment, as well as in the flower, on application of aluminium salts to the soil. In pink-flowered *Hydrangea* plants the original red colour of the stem spots and leaf-buds is changed to bright purple. This observation is in accordance with the general effect of aluminium on cyanidin derivatives. An inspection of the colour of stem spots and leaf-buds of plants in the dormant state would therefore provide a useful guide to importers and growers of alleged blue-flowering *Hydrangeas*.

Flowers damaged by frost also develop cyanidin derivatives, as was shown by the aluminium test and by Robinson and Robinson (1932). Yet another colour change occurs when *Hydrangeas* of all colours are grown in bright sunlight; this phenomenon usually appears about 2 weeks after the flowers have matured and is seen only on portions that are directly exposed to the sun, these becoming bright red in colour. The effect is very noticeable in white- and blue-flowered plants cultivated up to the flowering stage in a shaded greenhouse and then transferred to beds in full sunlight. The final colour of all *Hydrangea* flowers is green or greenish-yellow, but the above pigment persists very much longer than the normal pink or blue delphinidin derivatives.

The red pigment induced by sunlight, on testing with dilute aluminium and ferrous sulphate solutions, gives no colour change. However, the sodium acetate coloration being dull violet indicates the possibility of its being callistephin. If this proves the case, the *Hydrangea* during various stages of its existence must have synthesized pigments from all the three main anthocyanin groups: viz. pelargonidin, cyanidin, and delphinidin, together with anthoxanthins of unknown composition.

#### CULTURAL EXPERIMENTS

Attempts were made to produce pure blue flowers in the following plants, the petals of which contain delphinidin derivatives: *Campanula carpatica* (pH 6.4), *C. garganica* (pH 6.4), *Erinus alpinus*, *Nicotiana sieberi* (pH 5.2), *Linum grandiflorum* (red), *Primula obconica* (pH 6.3) red, and *Verbena* sp.

(crimson, pH 6.1). These plants were transferred at an early stage in their growth to soil containing aluminium phosphate and potash alum. Crystals of alum were placed on the surface of the soil, being renewed when dissolved. All species tested survived this treatment and many plants flowered successfully. In no instance did a blue colour or incipient blueness appear, despite the fact that aluminium was available over the whole of the growing period. Samples of leaves were analysed qualitatively for aluminium, but in no case was an abnormal accumulation found. Root analyses were not undertaken because previous experience with *Hydrangeas* had shown that such analyses may be untrustworthy because of the difficulty in removing adsorbed aluminium. The acidity of the flower saps of five of the experimental plants was considerably less (pH 5.2–6.4) than that of *Hydrangea* flowers (pH 4.4). This seems to suggest that cell-sap reaction is an important factor in aluminium absorption.

#### STUDIES OF ALUMINIUM-ACCUMULATING PLANTS

In 1934–5, when all the foregoing investigations were carried out, the number of aluminium-accumulating flowering plants known with certainty was only twenty-four, and most of the data regarding them was buried in obscure German and Japanese journals. At that time the writer could find in this literature (Yoshii and Jimbo, 1932; Neger, 1923) only three marked aluminium-accumulating families, viz. *Symplocaceae*, *Theaceae*, and *Diapensiaceae*, having missed the important works of Hallier (1922) and von Faber (1925). However, a close examination of these families revealed several facts bearing directly on the problem. This line of research (Chenery, 1936) eventually led to the discovery of seventeen new aluminium-accumulating species of seven new genera, but only one new aluminium-accumulating family, the *Diclidantheraceae* (now part of *Polygalaceae*); the cell-sap acidity range of these plants was shown to be pH 4.3–4.8.

#### THE PIGMENTS OF 'ALUMINIUM PLANTS'

*Flower pigments.* No blue-flowered plant was found amongst the above families. The nearest approach to blue occurs in the genus *Diclidanthera*, where the flowers are purple or purple-tinted. Assuming that the sap reactions of these plants fall within the range pH 4.3 to 4.8, the purple colour may be ascribed to the formation of a purple cyanidin-aluminium lake. One species of *Symplocos*, viz. *S. violacea*, has violet flowers possibly for the same reason as those of the *Diclidanthera* spp. *Symplocos* flowers are usually white, but are sometimes yellow and rarely pink.

Certain species of *Stuartia* also appear to exhibit the effect of aluminium in their flowers. In this genus the purple stamens of *S. malochodendron* and *S. pentagyna* indicate the presence of the cyanidin-aluminium lake.

*Fruit pigments.* Pigmented fruits are found very frequently in the *Symplocaceae*, rarely in the *Theaceae*, and not at all in the *Diapensiaceae*. The bright blue fruits of *Symplocos crataegoides*, the only species of the genus

hardy in England, at once recalled the blue of *Hydrangea* flowers and indicated a worthwhile avenue for further exploration. Brand (1901) in his monograph on the *Symplocaceae* describes the fruits as being black, brown, green, or sometimes yellow in colour. The fruits of *S. crataegoides* are well known to be ultramarine-blue in colour, but are described as black. Dried fruits of this species do actually appear very deep blue or blue-black, and since Brand compiled his monograph from herbarium material it is almost certain that the fruits of other species recorded as black were originally bright blue when fresh. In this category fall the fruits of the following species of *Symplocos*: *S. aegrota*, *S. altissima*, *S. angulata*, *S. colorata*, *S. coriacea*, *S. densiflora*, *S. furcata*, *S. macrostachya*, *S. mapiriensis*, *S. myrtacea*, *S. nivalis*, *S. prionophylla*, *S. rigidissima*, *S. setchuensis*, *S. siamensis*, *S. sinica*, *S. stawellii*, *S. tenuifolia*, *S. tristis*, *S. umbellata*.

The possibility that the blue fruits of *Symplocos* species owe their colour to neutral or alkaline cell-saps is precluded since the acidity of fruits is usually greater than the leaves of the same plant. It has been tacitly assumed in the foregoing considerations that all species of *Symplocos* are 'aluminium plants'; an assumption which will be justified below. This being so, the leaves of all *Symplocos* species would have a reaction in the 'aluminium-plant' range (pH 4.3-4.8), and it is probable that the blue fruits show almost the same reaction as the blue *Hydrangea* flowers, viz. pH 4.4.

The abundance of blue-fruited species in the *Symplocaceae* can hardly be a coincidence, and thus it might be confidently asserted that this blueness of the fruits is due to the presence of an acid stable aluminium-delphinidin lake, just as in the case of blue *Hydrangea* flowers.

*Leaf pigments.* Some species of the *Diapensiaceae* often display throughout the growing period a leaf pigmentation other than green. The colour is generally bright red at first, changing to purplish-crimson as the leaf ages. The leaves of *Galax aphylla* and *Schizocodon soldanelloides* are the best instances of this. It is possible that part of the effect is due to the purple cyanidin-aluminium compound and part to organic co-pigmentation by the tanninoid substances present.

It is relevant to note at this stage the influence of aluminium on the leaves of *Hydrangea macrophylla*. In the cultural experiments it was repeatedly observed that when the plants were treated with an excess of aluminium salts the leaves very soon turned yellow. The healthy leaves of both the artificially blue and naturally blue *Hydrangea* plants are affected in the same manner. Molisch (1922) also recorded the tendency of *Hydrangea* leaves to turn yellow after large applications of aluminium sulphate. This effect is in accordance with the formation of a deep yellow compound between aluminium and anthoxanthins as described previously.

A well-known characteristic of the leaves of species of *Symplocos* is their yellow or yellowish-green colour when dry. An examination of most of the 300 species as herbarium specimens in the British Museum (N.H.) revealed that the leaves were almost invariably coloured in this way. The same feature

can be seen in the leaves of several members of the Diapensiaceae. It was found recently that Hallier (1922) had made similar observations. One of the exceptions to the yellowish-green leaf-aluminium correlation<sup>1</sup> is *S. martinicensis*, which has chocolate-brown leaves in the dry state and very bright blue fruits.

#### BLUE FRUITS AND ALUMINIUM UPTAKE

While mapping the forest soils of Trinidad in 1937 blue-fruited plants were frequently seen and when tested for aluminium gave strongly positive results. The most conspicuous of these were plants of the Rubiaceae: *Coccolypselum guianense*, *Cephaelis pubescens*, *C. tomentosa*, and *Psychotria cuspidata*, and of the Melastomataceae: *Clidemia hirta* and *C. pustulata*. These plants only grow in the most acid soils in which readily available aluminium is present, consequently no pink- or red-fruited forms were ever seen. Here the search for 'Hydrangea-like' plants rested for several years.

It was not until the end of 1945 that the search was seriously resumed, after reading Hutchinson (1943) on the biogeochemistry of aluminium. In this article a very complete account of the occurrence and significance of aluminium and related elements in the plant, animal, and mineral world was presented, including several analyses of new or previously suspected aluminium-accumulating spermatophytes, the number being raised from 41 to 58. The work of Hutchinson has brought the importance of biogeochemical studies to the notice of English-speaking scientists and stimulated a resumption of the search for 'Hydrangea-like' plants and a much more comprehensive survey and study of 'aluminium plants'.

The first step then was to examine all the available members of the families Rubiaceae and Melastomataceae for new aluminium-accumulating species. A rapid and simple test was evolved (Chenery, 1946) and soon many new accumulators were brought to light. All the floras accessible in Trinidad were scrutinized for blue-fruited species. Leaf fragments of special species were obtained from the British Museum, the Chicago Natural History Museum, and from many botanists in the colonies, and in 1947 a systematic search was made in the Kew Herbarium. On the whole a very high correlation was found to exist between the presence of aluminium in abnormal quantities and a bright blue colour of the fruits of dicotyledons. There were exceptions, however, for together with 134 positive reactions 4 blue-fruited species failed to respond to the 'aluminon reagent' and another 16 species were only slightly above normal.

High aluminium uptake was not found in any blue-fruited species hardy in England except *Symplocos crataegoides*. Traces of aluminium somewhat above normal (about 400 p.p.m.) were found in *Gaultheria veitchiana*, *G.*

<sup>1</sup> In a recent survey in the Kew Herbarium of aluminium-accumulating dicotyledons made by the writer, 602 species from 136 genera gave yellowish-green (citron-green) leaves on drying. These comprised 37 per cent. of the total number of aluminium-accumulators found.

*sinensis*, *Elaeocarpus cyaneus*, and *Viburnum davidii*, but very little was found in *Gaultheria trichophylla*.

The exceptions to the correlation between blue fruit and aluminium accumulation do not detract much from its value in the search for 'Hydrangea-like' plants. It was found (Chenery, 1936) that as little as 11 p.p.m. of aluminium would change a solution of the pink delphinidin pigment to blue, and in the *Hydrangea* plant itself the minimum difference between a pink and blue flower was only 14 p.p.m. on a fresh-weight basis. That the blue colour of the fruits of the weakly accumulating and non-accumulators is an aluminium lake is still highly probable as the sap acidities of these plants are within the range pH 3.6–5.3, at which any normal anthocyanin is usually pink or red.

Co-pigmentation by anthoxanthins is not likely to be an important factor in the stabilization of pigments in the acid cell-saps of blue fruits as Robinson (1939) actually found less anthoxanthin in blue *Hydrangea* flowers than in the pink. Stabilization by organic colloids may possibly occur (Robinson, 1939), but in view of the good correlation between high aluminium content and blue fruits it would seem that inorganic aluminium provides the stabilizing factor. The possibility that iron also affects these fruit pigments might be disposed of in the same way as in the blue *Hydrangea* problem (Chenery, 1937). Iron in plants has not yet been recorded in abnormal amounts; 30 species of calcifuges were analysed (Chenery, 1936) and gave a mean iron-content in their dry leaves of 183 p.p.m., with a range of 31–300 p.p.m., all of which is conceivably needed for nutrition purposes. The 'aluminon' reagent would readily detect any iron-accumulator by giving a deep purple colour. Out of more than 3,800 tests only 5 species gave this colour, and of these, 3 species were water plants which were undoubtedly contaminated with iron.

The experiments of von Faber (1925) with Javanese solfatara plants—plants growing round sulphurous mud-volcanoes—should be mentioned. He grew *Elaeocarpus punctatus* in both an exceptionally acid volcanic soil and an ordinary garden soil of Java. In the former it made excellent growth and absorbed aluminium to the extent of 19,600 p.p.m., but in the ordinary soil only a trace. Since the volcanic soil was far more acid than any other natural soil (pH 1.0) it would be reasonable to expect the non-accumulating *Elaeocarpus* spp. to accumulate aluminium if sufficient were available. Von Faber also tested *Gaultheria leucocarpa* and found that although it was grown in the same acid soil in association with other strong accumulators this element was not taken up into its leaves but concentrated in its roots. It is thus very likely that the blue-berried species of *Gaultheria* absorb aluminium in sufficient quantities to produce the blue-delphinidin-lake. *Billardiera longiflora* and *Coprosma acerosa*, familiar to British horticulturists, absorb aluminium to just above the average for calcifuges, and also have high cell-sap acidities. In view of the above, their blue-fruit pigment is almost certain to prove a delphinidin-aluminium-lake. These plants would be worth testing

for fruit colour change, for they are more likely to survive aluminium-free conditions than a strong aluminium-accumulator.

Determinations of the cell-sap acidities of species of blue-fruited Trinidad plants widened the range found in England from pH 3·6 to pH 5·3. Maceration experiments were also performed on the blue and purple fruits, using aluminium sulphate solution and buffer solutions. No change in colour took place with the aluminium solution, and the blue pigments showed no signs of reddening until an acidity pH 3·5 was reached. Similar results were obtained with fruit pigments extracted with acetic acid and ether (these extracts were initially the same blue colour as in the fruits). Berries from a few species were matched against the R.H.S. Horticultural Colour Chart giving the following:

<i>Coccocypselum guianense</i>	.	.	.	.	Moorish blue (full hue)
<i>Cephaelis pubescens</i>	.	.	.	.	Spectrum blue (2nd and 3rd tints)
<i>C. tomentosa</i>	.	.	.	.	French blue (1st tint)
<i>Psychotria brachiata</i>	.	.	.	.	Gentian blue (full hue)

It must be stated here that some of the blue fruits of the Melastomataceae are really deep purplish-blue, possibly from admixture with cyanidin pigments, but the Rubiaceae fruits are royal or sky-blue in colour. Both the flesh and the skin of the fruits mentioned in this paper are blue. When petals of the pink Hydrangea and blue Chinese Myosotis were similarly examined the pigments were red or pink at pH 4·5–3·6 but changed to sky-blue on addition of the aluminium sulphate.

Blue-fruited monocotyledons<sup>1</sup> all proved to be non-accumulators. The species tested were: *Heliconia psittacorum*, *H. pulverulenta*, *H. bihai*, and an unidentified Canadian bog-plant. Their blue-fruit colour is probably due to the low cell-sap acidity which was found to be in the range pH 5·6–6·5. The sky-blue fruits of *Clerodendron fargesii* (Verbenaceae) also had an acidity of pH 6·0 and gave negative results with the 'aluminon' reagent.

Many of the new aluminium-accumulating species had bright purple-coloured fruits and some had purple leaves. Examples are listed in the appendix. Since the sap acidities of these plants are all in the range pH 3·6–4·6, the purple colour is certainly an acid-stable aluminium lake, this time of a cyanidin glycoside.

#### BLUE OR VARIABLE FLOWERS AND ALUMINIUM UPTAKE

During the search for new blue-fruited plants it was found that some of them were also blue flowering and that many other members of the same family were blue flowering but not blue fruiting, and that a few of the latter were even known to have flowers varying from pink to blue. These interesting plants belonged to the genera *Faramea*, *Palicourea*, *Manettia*, *Pentania*, *Congdonia*, *Sacosperma*, and *Borreria* (Rubiaceae) and *Brachyotum*,

<sup>1</sup> The only monocotyledons that have been found to accumulate aluminium in large amounts are the Rapateaceae, of which most genera dry a bright citron-green colour. The writer is indebted to Mr. N. Y. Sandwith, Kew, for drawing his attention to this family which proved wholly aluminium-accumulating.

*Pternandra* and *Memecylon* (Melastomataceae). If these plants were also aluminium accumulators, then they would be just the ones that would be most likely to fulfil all the requirements for the *Hydrangea* phenomenon. Leaves of many of these were obtained from the Chicago Natural History Museum, the British Museum, and Kew, and they all proved to be very strong aluminium accumulators.

*Faramea anisocalyx* was the most 'Hydrangea-like' species, for according to Standley (1936) it is reported by collectors in Peru as being covered with large bracts which are variously described as sky-blue, pink, or white. Dried herbarium specimens of this plant appear remarkably similar to those of *Hydrangea macrophylla*.

Since *Faramea anisocalyx* is amongst the strongest aluminium accumulators found so far, every endeavour should be made to obtain seeds of this beautiful shrub for the necessary cultural trials.

The genera *Palicourea* and *Memecylon* afford at least five species resembling *Hydrangea macrophylla* in flower-colour variability, if not changeability. These are all strong aluminium-accumulators, but not so strong as the *Farameas*. It is possible that this colour variability is due to differences in aluminium uptake, but at present no analytical data are available to support this, except leaf analyses of random samples. These plants are:

*Palicourea alpina* of Jamaica, which has flowers that are recorded by Fawcett and Rendle (1936) as being coloured red, yellow, purple, blue, or white.

*P. angustifolia*. Standley (1930) describes the flowers of this Colombian species as purple, violet-purple, violet, and pink.

*P. nigricans*. Standley (1936) describes the flowers of this species as dark blue, but Williams (1936) says it has flowers which vary from rose to violet.

*Memecylon membranifolia*. An African species described by Hutchinson and Dalziel (1936) as having pink or blue flowers.

*M. myrsinoides*. Ridley (1924) describes this Malayan plant as having rose-pink or pale blue flowers.

Over-optimism must be guarded against, even at this late stage in the search for 'Hydrangea-like' plants, for there is a possibility that these aluminium-accumulating blue or variable flowering species may fail to grow in the absence of aluminium. The experiments of Neger (1923) on *Symplocos japonica* showed that aluminium was essential for healthy growth. Later work by von Faber (1925) and Yoshii (1928) demonstrated that aluminium was also essential to solfatara plants. In spite of this it might be possible to cut down the aluminium to a very low level, sufficient for good growth but insufficient to reach the concentration in the flowers or bracts necessary for blue-lake formation.

There is another type of aluminium-accumulating effect that has not yet been studied; this is the absorption of aluminium not as cations from acid

media but as anions from strongly alkaline media. The number of species that will tolerate strongly alkaline conditions is very limited; this being so, the possibilities are very remote of finding one that displays a flower-colour change with differential aluminium uptake. Allen (1943), however, actually produced blue hydrangea flowers by growing the plants in a strongly alkaline soil (pH 7.5) treated with aluminium phosphate. The plants became extremely chlorotic and many died during the first growing season.

From this paper, which must be regarded as a progress report, it is clear that the search for 'Hydrangea-like' plants has been by no means exhaustive. The possible existence of many other such plants is therefore not unlikely. *Hydrangea macrophylla* still enjoys its singular position in the plant world chiefly because of the rarity of its suspected rivals and its tolerance to a wide range of soil reactions. Once collectors become what may be called 'Hydrangea conscious', some very interesting facts regarding the *Farameas* and *Palicoureas* will be brought to light. What is of prime importance in this connexion is a study *in situ* of the soil requirements of *Faramea anisocalyx*, and also analyses of its blue and pink bracts.

It may be concluded from all the evidence submitted above that there is now a probability that 'Hydrangea-like' plants do actually exist. Cultural experiments continued over several years with soils or nutrient media of high and low available aluminium content will be necessary to determine whether the *Hydrangea* flower-colour change is really unique.

#### SUMMARY

The possibility of producing in other plants colour changes similar to those occurring in the flowers of *Hydrangea* is discussed and a search for such plants has been conducted on the following lines. As many plants as possible were examined for the presence of the *Hydrangea* flower pigments (delphinidin glycosides) and seven of these were grown in the presence of excess aluminium but no change of flower colour was produced. This was probably due to the inability of these plants to translocate the aluminium because of too low cell-sap acidity. The literature concerning all the plants that were previously known or discovered during this investigation to accumulate aluminium in large amounts was examined in order to find blue-flowering species, and ultimately forty-one such plants were brought to light.

An interesting correlation has been established between the presence of bright blue fruits and aluminium accumulation. Out of 154 blue-fruited species 87 per cent. proved to be strong aluminium-accumulators. Evidence is adduced to show that the blue fruits of the few exceptions to this correlation owe their blue colour to the same pigment, namely, an acid-stable aluminium-delphinidin lake. The existence of purple-coloured, acid-stable cyanidin-aluminium lakes has also been revealed.

Of the blue-flowered aluminium-accumulators the most 'Hydrangea-like' species is *Faramea anisocalyx* followed closely by *Palicourea alpina* and *P. nigricans*.

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## APPENDIX

*Experimental*

The qualitative test for aluminium in plant material is described elsewhere (Chenery, 1946) and details of the method for quantitative determinations will be published in the near future. In the tables the approximate amounts of aluminium in the dry leaves are indicated as follows:

above 10,000 parts per million	+++
3,000–10,000   "   "   "	++
1,000–3,000   "   "   "	+
300–1,000   "   "   "	±
0–300   "   "   "	—

Cell-sap acidities were determined on the aqueous extracts of fresh crushed leaves by means of the quinhydrone electrode for those samples tested in England; the Trinidad samples were tested colorimetrically with indicators. All material examined, other than that from plants grown in England or Trinidad, was from dry herbarium specimens. pH tests on these were carried out quite easily by grinding the leaf fragments in an agate mortar with about 0.5 ml. of distilled water and testing the extract with indicators on a white tile. Previous checks with fresh or dry leaves of the same species gave practically identical acidities if the dry leaves were thoroughly ground.

TABLE I

*Plants with Delphinidin-aluminium Lakes*

## BLUE FRUITS

	Al. pH (p.p.m.)		Al. pH (p.p.m.)
Monimiaceae		Melastomataceae	
<i>Mollinedia caudata</i> Macbr. . . .	+	<i>Allomorphia procura</i> Craib . . .	+++
Lauraceae		<i>Dalelia pulchra</i> Korth. . . .	+++
<i>Litsea macleurei</i> Merr. . . .	+++	<i>Anplectrum cyanocarpum</i> Tri. . .	+++
Proteaceae		<i>A. glaucum</i> Tri. . . .	+++
<i>Helicia cochinchinensis</i> Lour. . .	+++	<i>Platycentrum clidmioides</i>	
<i>H. ferruginea</i> F.v.M. . . .	++	Naud. . . .	4.2 +++
<i>H. glabiflora</i> F.v.M. . . .	+++	<i>Leandra chaetodon</i> (S. & M.)	
Pittosporaceae		Cogn. . . .	4.6 +++
<i>Billardiera longiflora</i> Lab. . . .	4.8 ±	<i>L. costaricensis</i> (Don) Cogn. .	4.1 +
Theaceae		<i>L. longicoma</i> Cogn. . . .	4.4 ++
<i>Eurya chinensis</i> R. Br. . . .	+++	<i>L. rufescens</i> (DC.) Cogn. . .	3.8 +++
<i>E. distichophylla</i> Hemsl. . . .	+++	<i>Conostegia xalapensis</i> (Bpl.)	
<i>E. macartneyi</i> Champ. . . .	+++	Don . . . .	+++
		<i>Tetrazygia discolor</i> DC. . . .	+++

TABLE I (cont.)

	Al.			Al.	
	pH	(p.p.m.)		pH	(p.p.m.)
<b>Melastomataceae (cont.)</b>			<b>Loganiaceae (cont.)</b>		
<i>Miconia affinis</i> DC. . . . .	3.8	+++	<i>G. psychotrioides</i> Bak. . . . .	..	+++
<i>M. coerulea</i> Naud. . . . .	..	+++	<i>G. rosea</i> Thw. . . . .	..	+++
<i>M. cyanocarpa</i> Naud. . . . .	..	+++	<i>G. walkeri</i> Wight . . . . .	..	+++
<i>M. impetolaris</i> Don . . . . .	..	+++	<b>Rubiaceae</b>		
<i>M. laevigata</i> DC. . . . .	3.7	+++	<i>Coccocypselum canescens</i> Willd. . . . .	..	+++
<i>M. lilacina</i> Tri. . . . .	..	+++	<i>C. condalia</i> Pers. . . . .	4.4	+++
<i>M. nervosa</i> (Sm.) Tri. . . . .	3.8	9160	<i>C. guianense</i> (Aubl.) K. Sch. . . . .	4.3	5810
<i>M. prasina</i> DC. . . . .	3.8	+++	<i>C. guianense</i> (fruits) . . . . .	4.3	1810
<i>M. puberula</i> Cogn. . . . .	..	+++	<i>C. herbaceum</i> Aubl. . . . .	4.4	+++
<i>M. theazans</i> Cogn. . . . .	4.0	+++	<i>C. hirsutum</i> Bartl. . . . .	4.8	+++
<i>Clidemia capillaris</i> Gris. . . . .	4.0	+++	<i>C. lanceolatum</i> (R. & P.) Pers. . . . .	4.2	+++
<i>C. debilis</i> Crueg. . . . .	3.8	+++	<i>C. pseudotontanea</i> Gris. . . . .	4.4	+++
<i>C. dentata</i> Don . . . . .	3.9	+	<i>Bertiera guianensis</i> Aubl. . . . .	4.4	280
<i>C. dependens</i> Don . . . . .	..	++	<i>B. zaluzania</i> Gaert. . . . .	3.5	+
<i>C. deppeana</i> Standl. . . . .	..	++	<i>Craterispermum gracile</i> Chev. . . . .	..	++
<i>C. hirta</i> Don . . . . .	3.8	++	<i>Duidania montana</i> Standl. . . . .	4.6	+
<i>C. involucrata</i> DC. . . . .	3.8	++	<i>Faramea axillaris</i> Standl. . . . .	4.6	32100
<i>C. pustulata</i> DC. . . . .	3.8	7910	<i>Psychotria brachiata</i> Sw. . . . .	4.7	15400
<i>C. septiplinervia</i> Cogn. . . . .	..	+	<i>P. campylopoda</i> Standl. . . . .	4.6	+++
<i>Ossaea heteroneura</i> (Naud.)			<i>P. costaricensis</i> Polak . . . . .	5.2	+++
Tri. . . . .	4.5	+++	<i>P. cuspidata</i> Bred. . . . .	4.5	+++
<i>O. microphylla</i> Tri. . . . .	3.9	+++	<i>P. dispersa</i> Standl. . . . .	5.0	+++
<i>Pternandra coerulescens</i> Jack . . . . .	..	+++	<i>P. emetica</i> L. . . . .	5.0	690
<i>Memecylon arnottianum</i> Wht. . . . .	4.0	+++	<i>P. falcata</i> Rusby . . . . .	4.8	+++
<b>Elaeocarpaceae</b>			<i>P. involucrata</i> Sw. . . . .	4.4	+++
<i>Elaeocarpus cyaneus</i> Sims . . . . .	4.1	+	<i>P. iquitosensis</i> Standl. . . . .	4.8	+++
<i>E. ganitrus</i> Roxb. . . . .	4.1	+	<i>P. longirostris</i> (Rusby) Standl. . . . .	4.8	++
<i>E. holopetalus</i> F.v.M. . . . .	4.1	+	<i>P. patens</i> Sw. . . . .	4.5	+++
<i>E. hookerianus</i> Raoul . . . . .	..	-	<i>P. pittieri</i> Standl. . . . .	4.6	+
<i>E. obovatus</i> Don . . . . .	4.1	+	<i>P. rhodothamna</i> Standl. . . . .	5.0	+++
<b>Escalloniaceae</b>			<i>P. simiarum</i> Standl. . . . .	4.5	+++
<i>Polyosma integrifolia</i> Bl. . . . .	..	+++	<i>P. virgata</i> R. & P. . . . .	4.2	+++
<b>Ericaceae</b>			<i>Palicourea vestita</i> Standl. . . . .	4.4	9100
<i>Gaultheria sinensis</i> Auth. . . . .	..	+	<i>Rudgea justicoides</i> Standl. . . . .	4.6	36800
<i>G. tetramera</i> W. W. Sm. . . . .	..	+	<i>Chasalia capitata</i> DC. . . . .	..	+
<i>G. trichophylla</i> Royle . . . . .	..	-	<i>Geophila obvallata</i> Didr. . . . .	4.0	+
<i>G. veitchiana</i> Craib . . . . .	..	+	<i>Cephaelis alba</i> (Aubl.) Willd. . . . .	4.4	+++
<b>Symplocaceae</b>			<i>C. angustifolia</i> Ridl. . . . .	..	+
<i>Symplocos brandiana</i> K. & G. . . . .	..	+++	<i>C. axillaris</i> Sw. . . . .	4.6	+++
<i>S. costaricensis</i> Hemsl. . . . .	..	+++	<i>C. barcellana</i> (M.-A.) Standl. . . . .	4.6	+++
<i>S. crataegoides</i> Ham. . . . .	4.8	6560	<i>C. blephorophora</i> Standl. . . . .	4.9	+++
<i>S. curtisii</i> Oliv. . . . .	..	+++	<i>C. elata</i> Sw. . . . .	..	+++
<i>S. ferruginea</i> Roxb. . . . .	..	+++	<i>C. potaroensis</i> Sandw. . . . .	..	+++
<i>S. Martinicensis</i> Jacq. . . . .	4.3	+++	<i>C. pubescens</i> Britt & Standl. . . . .	4.6	+++
<i>S. myrtaea</i> S. & Z. . . . .	..	35400	<i>C. rosea</i> Bth. . . . .	5.0	+++
(Yoshii & Jimbo (1932))			<i>C. salicifolia</i> H. & B. . . . .	4.7	+++
<i>S. periangiana</i> K. & G. . . . .	..	+++	<i>C. singaporensis</i> Ridl. . . . .	..	+
<i>S. racemosa</i> Roxb. . . . .	..	+++	<i>C. tomentosa</i> Willd. . . . .	4.4	9950
<i>S. rigida</i> Clarke . . . . .	..	+++	<i>C. tontaneoides</i> Britt. & Standl. . . . .	4.8	+++
<b>Loganiaceae</b>			<i>C. violacea</i> (Aubl.) Willd. . . . .	4.6	+++
<i>Gaertnera crassiflora</i> Boj. . . . .	..	+++	<i>Lasianthus attenuatus</i> Jack . . . . .	..	+++
<i>G. koenigii</i> Wight. . . . .	..	+++	<i>L. batangensis</i> K. Sch. . . . .	..	+++
<i>G. pendula</i> Boj. . . . .	..	+++	<i>L. biernannii</i> King . . . . .	..	+++
			<i>L. cereiflorus</i> Bruce . . . . .	..	+++

TABLE I (cont.)

	Al. pH (p.p.m.)		Al. pH (p.p.m.)
Rubiaceae (cont.)			
<i>L. chinensis</i> Bth. . . . .	4.4 +++	<i>L. stipularis</i> Bl. . . . .	.. +++
<i>L. cyanocarpoides</i> Schl. . . . .	.. +++	<i>L. wallacei</i> Bruce . . . . .	.. +++
<i>L. cyanocarpus</i> Jack . . . . .	.. +++	<i>Saprosma ceylanicum</i> Bed. . . . .	.. +++
<i>L. fordii</i> Harris . . . . .	.. +++	<i>S. consimile</i> King . . . . .	.. +++
<i>L. filiformis</i> K. & G. . . . .	.. +++	<i>S. indica</i> Dalz. . . . .	.. +++
<i>L. herri</i> Craib . . . . .	.. +++	<i>S. pubescens</i> Ridl. . . . .	.. +++
<i>L. koi</i> Merr. . . . .	.. +++	<i>Amaracarpus caudatus</i> Ridl. . . . .	.. +++
<i>L. lanceolatus</i> (Gris.) Maz. . . . .	.. +++	<i>Coprosma acerosa</i> A. Cunn. . . . .	4.1 7
<i>L. lowianus</i> K. & G. . . . .	.. +++	<i>C. propinqua</i> A. Cunn. . . . .	4.1 7
<i>L. maingayii</i> H.f. . . . .	.. +++	<i>Didymaea alsinoides</i> (Met.) Standl. . . . .	5.1 —
<i>L. microcalyx</i> K. Sch. . . . .	.. +++	<i>Stachyococcus adenanthus</i> Standl. . . . .	.. +++
<i>L. nervosus</i> K. & G. . . . .	.. +++	Caprifoliaceae	
<i>L. oblongus</i> K. & G. . . . .	.. +++	<i>Viburnum davidii</i> Franch. . . . .	5.3 7
<i>L. pilosus</i> Wt. . . . .	.. +++	Verbenaceae	
<i>L. rhinocerotis</i> Bl. . . . .	.. +++	<i>Clerodendron fargesii</i> Dode . . . . .	6.0 —
<i>L. scabridus</i> King . . . . .	.. +++		
<i>L. seseensis</i> Tay. . . . .	.. +++		

TABLE II

## Blue Fruits and Blue Flowers

	Al. pH (p.p.m.)		Al. pH (p.p.m.)
<i>Manettia lygistum</i> (L.) Sw. . . . .	4.0 +	<i>Faramea quinqueflora</i> P. & E. . . . .	4.6 +++
<i>Faramea eurycarpa</i> Donn. Sm. . . . .	4.4 35000	<i>Psychotria herzogii</i> Moore . . . . .	4.6 22600
<i>F. rectinervia</i> Standl. . . . .	4.6 16000	<i>Memecylon coeruleum</i> Jack . . . . .	.. +++

## BLUE FLOWERS

<i>Hydrangea macrophylla</i> DC. . . . .	4.4 13000	<i>F. maynensis</i> Spruce . . . . .	4.5 +++
<i>H. macrophylla</i> (pink flowers) . . . . .	4.4 60	<i>F. salicifolia</i> Presl. . . . .	4.9 +++
<i>Sacosperma parviflorum</i> (Bth.) Tay. . . . .	.. ++	<i>F. stenura</i> Standl. . . . .	4.8 +++
<i>Pentania angustifolia</i> Hochst. . . . .	.. +++	<i>F. talamancarum</i> Standl. . . . .	4.7 +++
<i>P. pentagyna</i> K. Sch. . . . .	.. +++	<i>Palicourea dives</i> Standl. . . . .	4.7 +++
<i>P. procumbens</i> H. Good . . . . .	.. +++	<i>P. obtusa</i> (R. & P.) DC. . . . .	4.9 +++
<i>P. prunelloides</i> Kl. . . . .	.. +++	<i>P. polyneura</i> Kr. . . . .	4.7 +++
<i>P. schweinfurthii</i> Hiern . . . . .	.. +++	<i>Congdonia coerulea</i> (Gdn.) M.-A. . . . .	.. +
<i>Faramea amicalyx</i> P. & E. . . . .	4.6 36900	<i>Borreria dibrachiatia</i> Oliv. . . . .	.. +++
<i>F. brachysiphon</i> Standl. . . . .	5.0 +++	<i>Brachyotum maximowiczii</i> Cogn. . . . .	.. +++
<i>F. cestroides</i> Standl. . . . .	4.7 +++	<i>Memecylon obiculare</i> Thw. . . . .	4.0 +++
<i>F. cuspidata</i> Benth. . . . .	5.0 +++	<i>M. polyanthemus</i> H.f. . . . .	4.0 +++
<i>F. glandulosa</i> P. & E. . . . .	4.8 +++	<i>M. spathandra</i> Bl. . . . .	4.0 +++
<i>F. insignis</i> Standl. . . . .	4.6 40000	<i>M. strychnoides</i> Bak. . . . .	4.0 +++
<i>F. kalliipii</i> Standl. . . . .	4.7 +++	<i>M. varians</i> Thw. . . . .	4.0 +++
<i>F. longifolia</i> Bth. . . . .	4.8 +++	<i>Pternandra capitella</i> Jack . . . . .	.. +++
		<i>Utricularia angustifolia</i> Benj. . . . .	4.6 3460

## VARIABLE FLOWERS

<i>Palicourea alpina</i> (Sw.) DC. . . . .	4.2 +++	<i>Memecylon membranifolium</i> H.f. . . . .	4.0 +
<i>P. angustifolia</i> H.B.K. . . . .	.. +++	<i>M. myrsinoides</i> Bl. . . . .	.. +++
<i>P. nigricans</i> Kr. . . . .	4.6 17100		

TABLE II (cont.)  
PLANTS WITH CYANIDIN-ALUMINIUM LAKES

PURPLE FLOWERS OR STAMENS			
	Al.		Al.
	pH (p.p.m.)		pH (p.p.m.)
<i>Stuartia malochodendron</i> L. . . .	10800	<i>Dissotis cornifolia</i> H.f. . . .	3·8 +++
<i>S. pentagyna</i> L'Her. . . .	3530	<i>D. grandiflora</i> Bth. . . .	4·0 +++
<i>Dididanthera elliptica</i> Miers . . .	7350	<i>D. Irvingiana</i> H.f. . . .	3·6 +++
<i>D. laurifolia</i> Mart. . . .	3900	<i>D. trothae</i> Gilg. . . .	+++
PURPLE FRUITS			
<i>Miconia acinodendron</i> (L.) Tri. . .	3·8 66100	<i>Henriettea succosa</i> (Aubl.) DC. .	3·8 21900
<i>M. racemosa</i> (Aubl.) DC. . . .	3·8 +++	<i>Palcourea crocea</i> (Sw.) R. & S. .	4·4 9300
<i>Clidemia crugeriana</i> Gris. . . .	3·8 +++	<i>Cephaelis mucosa</i> Sw. . . .	4·3 13700
PURPLE LEAVES			
<i>Miconia ciliata</i> (Rich.) DC. . . .	3·8 16500	<i>Schizocodon soldanelloides</i>	
<i>Galax aphylla</i> L. . . .	4·6 12000	S. & Z. . . .	7590
		Mean aluminium content of 22	
		calcifuges of non-accumulat-	
		ing families . . . .	206

#### ADDITIONAL NOTES

In the above genera there are probably very many more blue-fruited species that have not yet been tested. The number of aluminium-accumulators has been raised from 44 to 1,600 by this investigation; a list of genera will be published elsewhere.<sup>1</sup>

*Meconopsis betonicifolia* is mentioned in the short article in 'Nature' on this subject (Chenery, 1946). The present writer was not responsible for the inclusion of this species, which in its context might be considered 'Hydrangea-like'. This, however, is not the case as the *Meconopsis* flowers do not contain delphinidin, neither do the plants absorb aluminium (negative 'aluminum' reactions were obtained with both English and Tibetan specimens). There is a variability in the colour of *Meconopsis* flowers, but it is not from sky-blue to bright pink as obtains in *Hydrangeas* but from sky-blue to dull shades of lilac. Robinson (1939) found that the lilac petals had the same acidity as blue ones but contained more anthocyanin. The increase in anthocyanin content may have been caused by too much sunlight or from genetic deterioration of the strain; how far soil differences are responsible has not yet been ascertained.

Maceration tests with pink *Hydrangea* petals and nitrates of zirconium, lanthanum, and thorium disclosed that only the first element gave bright blue colorations. Lanthanum gave purple colorations, thorium had no effect. Individual flowers absorbed the zirconium too slowly to produce blue colours in the petals but the pink pedicels turned blue overnight at their tips. Zirconium is an extremely rare element except in very insoluble minerals, and so is not very likely to affect flower colours in Nature.

<sup>1</sup> Every dicotyledonous family in the Kew Herbarium was tested for aluminium and apart from those recorded above, accumulators were only found in the following: *Violaceae*\*, *Polygalaceae*\*, *Vochysiaceae*\*, *Scytopetalaceae*\*, *Icacinaeae*\*, *Octoknemataceae*\*, *Phyllonomaceae*\*, *Cunoniaceae*\*, *Rhizophoraceae*\*, *Crypteroniaceae*\*, *Gentianaceae*\*, *Geisolomataceae*\*, *Myrsinaceae*\*, *Euphorbiaceae*, *Dalphiniphyllaceae*\*, *Juglandaceae*. New records are starred.

# The Continuous Swelling of Brown Algae

BY

ALBERT WASSERMANN

With three Figures in the Text

THE continuous or unlimited swelling of brown algae occurs in aqueous solutions of electrolytes to be specified below and there are swelling maxima within a fairly narrow concentration range. In contrast to the limited swelling<sup>1</sup> a relatively large amount of water is taken up, this being followed by an irreversible conversion of the cell tissue into a soft pulp and by the formation of a solution of markedly increased viscosity, which is due to the dissolution of an alginate. No previous systematic investigation of this peculiar gel-sol transition appears to have been made, although the effect has been noted by various authors (see, for instance, Kohler, loc. cit., or Miwa, 1940). An attempt is now made to find out by qualitative observations, rather than by quantitative analytical methods, whether this type of swelling is due to heterogeneous ionic exchange processes.

## EXPERIMENTAL PART AND RESULTS

Discs or strips were cut from the fronds of mature algae, the stipes or holdfasts not being investigated. Control tests were done with material cut from various parts of the fronds and from algae collected at different parts of the coast and in different seasons; these measurements indicate that the unlimited swelling is not markedly dependent on the history of the test pieces. The pretreatment of the algal material involved either a thorough wash with distilled water, or an extraction with an excess of 1 N hydrochloric acid, for about half an hour (at room temperature) followed by another water wash. The pretreated test pieces, in which the majority of the cells were dead, were suspended at  $20 \pm 2^\circ$ , for known time intervals, in the swelling agents and were then drained for 2-3 minutes on a perforated glass plate, the final weighing operation being done in glass boats with ground-on covers. The swelling is defined by  $\Delta = nW_t - W_o$ , where  $nW_t$  is the weight of a swollen disc or strip at time  $t$ ,  $W_o$  is the weight of the test piece fully saturated with water, before being brought in contact with the swelling agent, and  $n$  is the number of test pieces. The result of a typical experiment is in Table I.

<sup>1</sup> The limited swelling of brown algae and the reverse drying reactions have been frequently investigated: see, for instance, Reinke (1879), Pringsheim (1923), Kohler (1932, 1936), Isaac (1933, 1935), Zaneveld (1937), Biebl (1938), Kylin (1938).

TABLE I. Swelling of *Laminaria digitata* in 0.1 m. Sodium Carbonate Solution:  $W_0 = 0.26$  g.

Time in hours.	1	2.2	4.2	6.5	8	23
Percentage increase of weight of disc (mean value of four runs)	$27 \pm 30$	$246 \pm 20$	$304 \pm 20$	$390 \pm 23$	$422 \pm 30$	—
Mechanical property of disc	Cut edge becomes soft	Soft throughout		Soft pulp		Complete degeneration

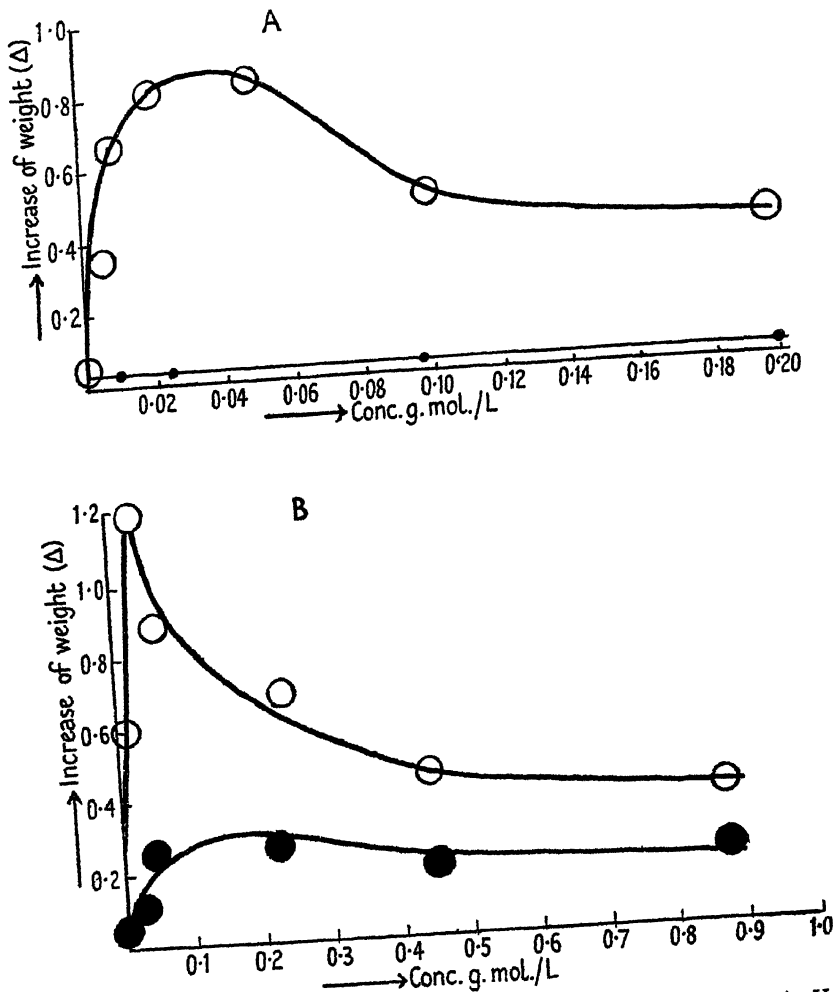


FIG. 1. The continuous swelling of *Laminaria digitata* (discs of 1.8 cm. diameter). Here and in the following figures the black and white circles relate respectively to the water and to the hydrochloric acid-extracted algal material A=NaOH solution, pH=11-13, 4 hours; B=Na<sub>2</sub>CO<sub>3</sub> solution, pH=11-12, 2 hours. Here and below  $\Delta$  is given in grammes.

The temperature dependence of the rate of swelling was tested with three species of algae and at four temperatures within  $0-40^{\circ}$ , the other conditions being as in the above experiments:  $\Delta_{t+10^{\circ}}/\Delta_t$  varied between 1.1 and 1.3. No influence of illumination or of the diameter of the discs on the rate of swelling could be detected, but stirring increased the speed of water uptake. The experiments were nevertheless done in a static system in order to avoid the breaking up of the swollen material into smaller particles. Parallel runs were carried out in which respectively oxygen and purified nitrogen were bubbled through the reaction mixture; it was found that the rate of swelling was not detectably different. In all experiments the volume of the reaction

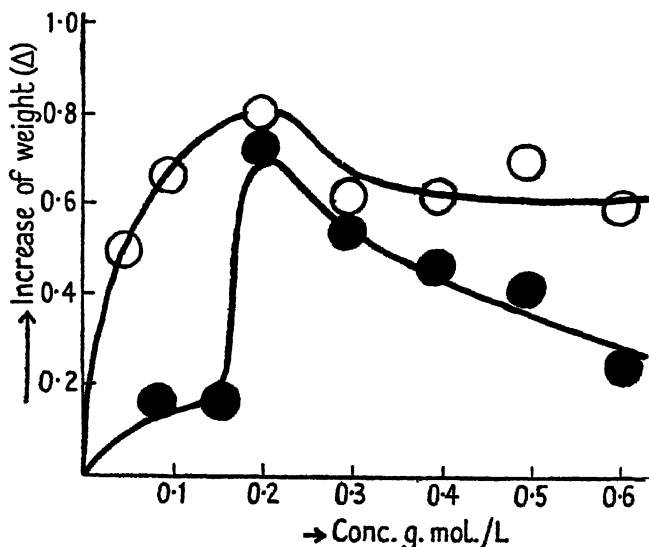


FIG. 2. Continuous swelling of *Laminaria digitata* discs (diameter 1.8 cm.) in varying concentrations of  $\text{Na}_2\text{HPO}_4$ , pH 9.5-10; black circles 6 hours, white circles 2 hours.

mixture was so large that the ratio of the number of equivalents of the swelling agent to the number of equivalents of alginic acid in the test piece was at least 50. The accompanying figures and the data in Table II demonstrate the influence of the pretreatment with 1 N hydrochloric acid and of the type and concentration of the swelling agent. As regards this latter variable, graph A in Fig. 1 shows, for instance, that with about 0.04 m. sodium hydroxide the continuous swelling of the algal material is more pronounced than with either more concentrated or more dilute solutions. Similar effects were observed in experiments with other algae and with other solutions (see the graphs in Figs. 2 and 3), the numerical values of the various 'maximum' swelling concentrations' being listed in the last columns of Tables II and III. The symbols + or - in these tables and in Table V indicate whether the continuous swelling in the relevant run was detectable or not. It will be seen that in some tests (e.g. with sodium hydroxide or sodium acetate solutions)

no continuous swelling of the water-pretreated algal material took place, while after treatment with hydrochloric acid the continuous swelling occurred.

TABLE II

*Continuous Swelling of Water and Hydrochloric Acid-pretreated Laminaria digitata*

Swelling agent.	Conc. range of swelling agent (g.-mol./l.).	pH range.	Pretreatment of algal material with.	Time of contact between algal material and solution (hrs.).	Continuous swelling.	Maximum swelling conc. (g.-mol./l.).
Sodium oxalate	0.01-0.3	8.5-9	H <sub>2</sub> O	1½	+	0.1
			HCl		+	0.1
Sodium fluoride	0.01-0.9	~8	H <sub>2</sub> O	3	+	0.1
			HCl		+	0.5
Sodium acetate	0.01-1.0	8.5-9.5	H <sub>2</sub> O	60	—	—
			HCl	1½	+	0.1

Potassium carbonate, phosphate, oxalate, fluoride and acetate or potassium and ammonium hydroxides produced similar effects to those brought about by the corresponding sodium compounds; solutions of alkali chlorides (pH=5-7) or of calcium or barium hydroxides (pH=13-14) did not give rise to continuous swelling under conditions similar to those specified above. The following species were also tested: *Laminaria saccharina*, *L. Cloustoni*, *Chorda filum*, *Ascophyllum nodosum*, *Fucus vesiculosus*, *F. serratus*. These experiments were done with the solutions of the substances listed in the second column of Table III. It should be noted that all the water-pretreated Laminariales reacted in a similar way, while the water-pretreated Fucales were more resistant; there was no marked difference, however, between the hydrochloric acid-extracted Laminariales and Fucales.

TABLE III

*Continuous Swelling of Water-pretreated Brown Algae*

Alga.	Solution contains.	pH range.	Time of contact between algal material and solution (hrs.).	Continuous swelling.	Maximum swelling concentration (g.-mol./l.).
Laminariales	NaOH	9.5-14	2-24	—	—
(4 species listed in text)	Na <sub>2</sub> CO <sub>3</sub>	11.5-12	2-24	+	0.1
	Na <sub>2</sub> PO <sub>4</sub>	12.3-13	2-24	+	0.01-0.05
<i>Ascophyllum nodosum</i>	NaOH	9.5-14	100	—	—
	Na <sub>2</sub> CO <sub>3</sub>	11.5-12	100	—	—
	Na <sub>2</sub> PO <sub>4</sub>	12.3-13	46	+	0.05
<i>Fucus vesiculosus</i>	NaOH	9.5-14	100	—	—
	Na <sub>2</sub> CO <sub>3</sub>	11.5-12	100	—	—
	Na <sub>2</sub> PO <sub>4</sub>	12.3-13	7	—	—
<i>Fucus serratus</i>	NaOH	9.5-14	7	—	—
	Na <sub>2</sub> CO <sub>3</sub>	11.5-12	100	—	—
	Na <sub>2</sub> PO <sub>4</sub>	12.3-13	7	—	—

At the end of each set of these tests the solution was separated from the residue and made equimolar with respect to the swelling agent. The viscosities

of the 'adjusted solutions' were then determined and found to be relatively large if a continuous swelling of the algae had taken place; if, on the other hand, no continuous swelling had occurred, the viscosity of the final solution was not larger than that observed in a control run in which the algal material had been suspended in distilled water. Typical results are in Table IV, the

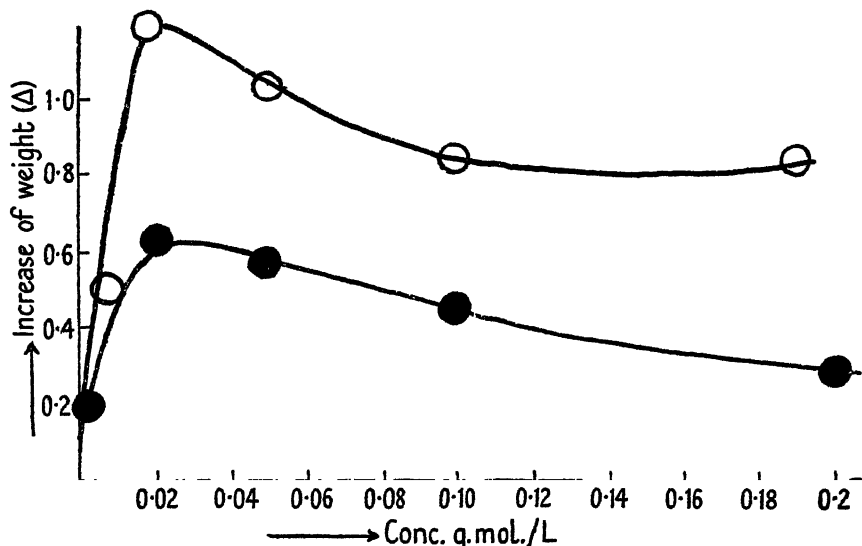


FIG. 3. Continuous swelling of *Laminaria digitata* discs (diameter 1.8 cm.) in varying concentrations of  $\text{Na}_3\text{PO}_4$ , pH 12-13; black circles 6 hours, white circles 2 hours.

test pieces being discs of 1.8 cm. diameter ( $W_0 = 0.20 \pm 0.02$  g.) and the swelling time being 2 hours. It should be noted that the viscosity of the adjusted filtrate is largest if the concentration of the swelling agent is 0.257 g.-mol./l. Similar viscosity maxima, at electrolyte concentrations between 0.02 and 0.3 g.-mol./l., could be also observed in most other experiments in which the continuous swelling took place.

TABLE IV

*Swelling and Viscosity Determinations done with Water-pretreated Laminaria saccharina and Sodium Carbonate as Swelling Agent*

Conc. of $\text{Na}_2\text{CO}_3$ solution in swelling test (g.mol./l.).	Percentage increase of weight of disc.	Conc. of $\text{Na}_2\text{CO}_3$ in adjusted solution, used for viscosity determination (g.mol./l.).	Viscosity (centistokes) at 25.0°.
1.03	70		1.107
0.515	185		1.112
0.257	195	0.502	1.184
0.103	215		1.129
0.0500	120		1.108
0.0250	65		1.109

Experiments with four species were done in which the acid-extracted algal

material was recalcified<sup>1</sup> by suspension in solutions of calcium chloride or calcium hydroxide, the conditions of concentration, &c., being similar to those indicated in a paper to be published elsewhere (Wassermann, 1947). The test pieces were then washed with water and placed for 2 hours in a solution of sodium hydroxide of pH=13.5, when no continuous swelling was detectable. The influence of recalcification on the continuous swelling of *Laminaria digitata* was further tested as indicated in Table V.

TABLE V

*Influence of Calcification on the Continuous Swelling of Laminaria digitata*

Pretreatment of algal material.	Continuous swelling in 0.05 N sodium hydroxide (pH=13.5) after contact times of 2 hrs.
Alga pretreated with water (A)	—
(A) Extracted with 1 N HCl and washed with water (B)	+
(B) placed in 0.01 N Ca(OH) <sub>2</sub> and washed with water (C)	—
(C) Extracted with 1 N HCl and washed with water (D)	+
(D) placed in 0.01 N Ca(OH) <sub>2</sub> and washed with water	—

## DISCUSSION

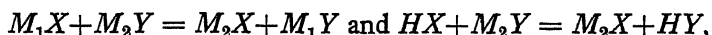
It has been shown (Wassermann, loc. cit.) that the fronds of brown algae contain alginic acid (*a*) and other acidic substances (*b*) in the form of water-insoluble salts, there being also other tissue constituents (*c*) which appear to be esters; and that on extraction with 1 N hydrochloric acid the salts are converted into free water-insoluble acids. Most swelling experiments were done at high pH values, when a rapid hydrolysis of (*c*) takes place, but it has also been found that the continuous swelling can be brought about at pH 7–8; in such solutions the esters are relatively stable, and it is concluded therefore that, in general, ester hydrolysis is not a necessary condition for the continuous swelling of these algae. The presence of sodium, potassium, or ammonium ions is of importance, on the other hand, for solutions containing calcium or barium do not give rise to continuous swelling, although the alkaline earth ions are powerfully absorbed. These observations are related to the results of experiments (Wasserman, loc. cit.) which showed that the sodium, potassium, or ammonium salts of (*a*) and (*b*) are water-soluble, while the calcium or barium salts are practically insoluble. It is believed, therefore, that the primary stage in the continuous swelling involves the following reactions:

Water-insoluble salts of (*a*) and (*b*) in algal tissue = Water-soluble salts in algal tissue (1)

Free water-insoluble acids (*a*) and (*b*) in algal tissue = Water-soluble salts in algal tissue (2)

<sup>1</sup> This term refers to the fact that prior to the extraction with hydrochloric acid the algal tissue contains a relatively large amount of calcium.

These are cation exchange processes of the type



where  $X$  and  $Y$  are anions,  $M_1$  and  $M_2$  are metal ions in the exchange positions, and  $H$  is the exchangeable hydrogen ion. The graphs in the figures, Table II, and the experiments mentioned on page 140, indicate that the hydrochloric acid-pretreated algal material swells more rapidly than the water-washed test pieces, some of which show no continuous swelling (see Table III). The different reactivity is believed to be due to the fact that the hydrochloric acid-extracted cell tissue contains the free acids ( $a$ ) and ( $b$ ), while the water-washed material contains salts of ( $a$ ) and ( $b$ ), the rate of conversion of the free acids being relatively large. The alteration of the swelling properties could be ascribed to some unknown process which may be taking place while the algal material is in contact with the hydrochloric acid. If this were the case it would be difficult to understand why the recalcification of the hydrochloric acid-extracted material alters the swelling properties in the way described on page 142. Some of the observations referred to in the preceding section show that the continuous swelling of a given algal material is influenced by the nature of the anion  $Y$ ; an effect of this kind is not incompatible with the assumption that a cation exchange plays a role, for it is known that such reactions are influenced by the nature of the anion (see, for instance, Nachod and Wood, 1945).

The small temperature coefficient of the continuous swelling and the observed influence of mechanical agitation of the reaction mixture indicate that the rate-determining stage of these processes does not involve the rupture of primary valence linkages, but is rather of the nature of a diffusion such as:

Water-soluble salts in algal tissue  $\rightarrow$  Water-soluble salts in solution. (3)

This reaction could produce cracks or holes in the cell tissue, which would be filled up with solvent, and this could contribute to the observed increase of weight of the algal material. In dilute solutions an increase of the concentration of the swelling agent will increase the amount of the soluble salts in the cell tissue, formed according to (1) or (2), and thus the rate of (3) and of the continuous swelling would increase. At substantially higher electrolyte concentrations, on the other hand, some of the soluble salts in the cell tissue could be salted out,<sup>1</sup> thereby blocking the channels through which diffusion normally takes place; this would slow down the rate of (3), which in turn would decrease the rate of swelling. There could be an intermediate concentration range, however, when salting-out effects are not yet operative, and under these conditions swelling maxima such as those described in the preceding section could obtain. An alternative explanation is as follows. The formation of the soluble salts according to (1) or (2) must be responsible for an osmotic pressure increase inside the algal tissue, which will give rise to an uptake of water. The swelling maxima could be due to the presence of a

<sup>1</sup> The salting out of sodium alginate by electrolytes is well known; compare Heen (1938).

membrane, permeable to low-molecular species, but impermeable to alginate or to other high-polymeric electrolytes, for the 'effective' osmotic pressure inside the membrane would be largest if the electrolyte concentration in the outside solution is neither too high nor too low (see, for instance, Freundlich, 1923). It has to be postulated, however, that the osmotically distensible membrane system of the algal material breaks down before a steady state is reached; this follows from the results of the viscosity determinations, which indicate that a water-soluble alginate diffuses into the outside solution, Rose (1937) and Heen (*loc. cit.*) having shown that dilute alginate solutions are relatively viscous. Thus the continuous swelling of these algae is a more complicated colloid-chemical process than the limited swelling of other biological materials (see Procter and Wilson, 1916, or Boyle and Conway, 1941), and no attempt has been made to measure the ionic adsorption by the degraded algal tissue in order to find out whether the swelling maxima can be interpreted from the standpoint of a Donnan equilibrium. It should be noted, however, that not only swelling maxima, but also viscosity maxima occur, if the concentration of the outside solution is within the range given in the last columns of Tables II and III; this is compatible with both explanations, and it is probable that the quantity of liberated alginate will be relatively large under conditions favourable for continuous swelling. The observed relation between swelling and viscosity is of interest because it indicates the optimum concentration conditions for the isolation of alginates from brown algae.

#### SUMMARY

The continuous or unlimited swelling of water or hydrochloric acid-pretreated brown algae occurs in solutions of specified cations and anions, there being swelling maxima within a fairly narrow concentration range. A number of qualitative tests are described which make it probable that the primary step involves an exchange adsorption in which metal or hydrogen ions are replaced by other cations.

The results described were selected from many more results of experimental work carried out for the Ministry of Home Security and for the Ministry of Supply at their Leamington Station. This paper is published with the approval of the Director General of Scientific Research (Defence), to whom thanks are expressed. I am further greatly indebted to a number of my colleagues at Leamington Spa, at the Marine Biological Station in Plymouth, and at University College, London, for many helpful discussions.

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## Studies in the Development of the Inflorescence

### V. The Raceme of *Lobelia Dortmanna* L., and other Campanulaceous Inflorescences<sup>1</sup>

BY

W. R. PHILIPSON

With Plate IV and two Figures in the Text

THE inflorescences of the Campanulaceae have attracted attention on account of their plasticity. Both Parkin (1914) and Goebel (1931) used examples chosen from this family to illustrate flower arrangements intermediate between the cyme and the raceme. *Lobelia Dortmanna* L. was chosen for study because its inflorescence is strictly racemose, but other Campanulaceous types that approach a cymose arrangement of their flowers were examined and one of these, *Campanula persicifolia* L., is described later in this paper. *Jasione montana* L. was also examined because its inflorescences are in the form of capitula.

#### I. *LOBELIA DORTMANNA* L.

*Lobelia Dortmanna* L. has long attracted notice because of its unusual habit and because of its restriction to water of low mineral content (Roll, 1939; Tansley, 1911, 1939; West, 1906, 1910). The main axis of the plant bears a submerged rosette of stiff leaves. In May this axis begins to elongate rapidly and forms the flower-stalk which projects above the surface of the water. After flowering, buds in the axils of the upper leaves of the rosette form new rosettes which survive the winter, but which do not appear to flower until they are 2 years old.

*The hydathodes.* The structure of the leaves has been described, particularly with regard to the two longitudinal air spaces and the hydathodes which occur at their tips (Armand, 1912; Buchenau, 1866; Minden, 1899; Tswett, 1907). Armand refers to the early appearance of these well-defined hydathodes, but I have seen no account of their development. As they occur in the bracts and floral organs as well as in the foliage leaves a brief description of their development is included here.

Pl. IV, Fig. 1, represents a longitudinal section through the apex of a rosette of *Lobelia* upon which the primordia of foliage leaves were appearing. A swelling to the right of the apex ( $I_1$ ) represents an early stage in the initiation of a leaf. To the left of the apex the rudiment of a leaf  $220\mu$  high is cut medianly ( $I_2$ ). This rudiment is in no way abnormal, resembling the form of leaf-rudiment found in many Dicotyledons by being curved towards the

<sup>1</sup> Part of a thesis approved for the degree of Ph.D. in the University of London, 1947.

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stem apex, by having an obtuse tip, and by showing a provascular meristem separated from the upper and lower epidermis by vacuolating tissue. The slightly older leaf-rudiment to the right of the figure ( $l_3$ ) is only  $300\ \mu$  high, and yet it differs considerably in form and structure from the leaf at  $l_2$ . It also is cut medianly, but the apex can be seen to have become dilated into a bulbous structure composed of enlarged and highly vacuolated epidermal cells enclosing a homogeneous tissue of dense rounded cells. These cells form the epithema of the hydathode, and the provascular meristem can be seen to end in contact with them.

An interesting feature of the cells of the epithema of this very young leaf is that they are slightly larger than the still immature cells of the other tissues of the leaf. In the mature leaf, on the other hand, the epithema cells are considerably smaller than the other tissues. A comparison of the size of the epithema cells of this young leaf and those of the considerably older leaf (in this case a bract) shown in Pl. IV, Fig. 3, which was photographed at the same magnification, shows that the epithema cells of the two leaves are approximately of the same size. We may therefore suppose that the epithema reaches maturity at a very early stage in leaf development. This supposition is supported by the highly magnified apex of a leaf-rudiment seen in Pl. IV, Fig. 2. The leaf to which this apex belonged was only  $400\ \mu$  high, and therefore scarcely older than that seen in the first figure, and yet in addition to the epithema cells being to all appearances mature, a stomata can be seen ( $s$ ) which corresponds with those figured by Tswett from the mature leaf. No doubt this early assumption of the mature condition is connected with the need for a translocation stream through the young leaves, a current that probably requires an active hydathode for its maintenance. It is of interest that, in addition to the well-defined hydathodes found in leaves, bracts, and sepals, similar but much smaller structures terminate the principal vascular strands of the petals, and possibly also of the young stamens and ovary.

The early transformation of the leaf-apex into a hydathode prevents further growth of the leaf. Subsequent growth is intercalary, being particularly active just below the epithema, cells being added not only to the leaf itself but also to the epithema, which therefore increases in size. As the leaf enlarges, other hydathodes are formed along the margin of the apex by the conversion of the cells to epithema and the development of stomata. An anastomizing system of vascular bundles develops in connexion with these separate hydathodes (Pl. IV, Fig. 3).

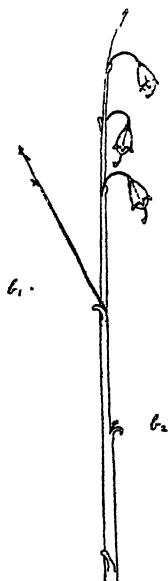
*The raceme.* The arrangement of the flowers is extremely simple. The stout axis of the rosette is continued as a slender scape on which the bracts continue the phyllotaxis of the leaves. The bud-meristems in the axils of the lower bracts form small protruding primordia similar to those in the axils of the foliage leaves, and like them they do not develop further, but remain as isolated pockets of meristem having no connexion with the vascular system of the axis. One or two of the buds in the axils of the upper foliage leaves are destined to form new rosettes. The behaviour of these buds was not studied

in detail, but they can be recognized by their large size and the presence of vascular strands connecting their prophylls with the stele of the main axis (Pl. IV, Fig. 4).

The development of the buds in the axils of the bracts immediately below the raceme is interesting. These buds are stimulated to develop further than those at the base of the scape. It is probable that the stimulus prompting this increased development comes from above, because the degree of development falls off towards the base of the plant. No doubt the stimulus is connected with the production of flowers which begins at the next higher node of the scape. The development, however, is not in the direction of a single flower, but towards a secondary axis bearing flowers in a racemose sequence. The development rarely proceeds very far, and the production of visible branches, as in the plant sketched in Text-fig. 1, is so rare as to be considered abnormal. The flower-buds borne on the lateral branches of this scape were rudimentary, even though the flowering of the main axis had been completed, and they probably would not have come to maturity.

The buds in the axils of the upper bracts develop into single flowers in the order of their initiation, that is, in acropetal sequence. Bracts and floral primordia continue to form until the entire apex of the scape is used up in forming the primordium of the last flower, which may therefore be regarded as terminal (Pl. IV, Fig. 5). There is no tendency for the terminal flower to come to maturity in advance of those below, the gradient of development remaining in the same direction as that of the growth of the axis and of the initiation of the primordia. Indeed, the later-formed flower-rudiments frequently fail to complete their development, withering away while still in the bud. The inflorescence therefore has all the characters of a true raceme.

The onset of flowering is first disclosed by an abrupt increase in the length of the internodes. The internodes between the foliage leaves remain extremely short (Pl. IV, Fig. 4), but those between the bracts elongate very considerably while the bracts are still rudimentary (Pl. IV, Fig. 6). This striking development of the internodes so close to the apex is no doubt related to the aquatic habitat of the plant. One result is that the scape projects freely into the water above the rosette while the flowers are still very young. In land plants with a similar habit the internodes of the flowering stem usually remain short until the flower-rudiments are well developed, and then elongate rapidly just before flowering (compare *Campanula persicifolia* L., described later in this



TEXT-FIGURE 1.  
*Lobelia Dortmanna*  
L. Drawing of the middle portion of a scape, showing the three lowest flowers of the raceme. The buds in the axils of the bracts below the raceme have formed branch-rudiments ( $b_1$ ,  $b_2$ ), the upper of which has elongated in an abnormal way.

paper, and *Agrostis canina* L. (Philipson, 1935)). A further indication of the onset of flowering is the absence of adventitious roots in the internodes between the bracts; in the longitudinal section of the vegetative apex shown in Pl. IV, Fig. 4, the initiation of adventitious roots can be seen to occur close behind the apex.

The development of the bracts is in all respects similar to that of the leaves. Their initiation close to the apex, as in the foliage leaves, is striking (Pl. IV, Fig. 6). The narrow apex of the inflorescence with the primordia of the bracts crowded around it is in contrast to that found in inflorescences with broad receptacles. This difference in organization appears to be due to the continued growth in length of the raceme. The anatomy of the scape is very simple, because each bract receives only a single trace.

The initiation and development of the floral buds is very similar to that of the buds of the branches of the inflorescence in *Valeriana* (Philipson, 1947a), except that the gradient of development continues in the same acropetal direction as that of their initiation (Pl. IV, Figs. 5 and 6). At the same time that each bract-primordium is initiated the portion of the apical meristem in its axil enlarges to form a floral primordium (Pl. IV, Fig. 7). This primordium retains meristematic connexions with the provascular meristem on either side of the gap above the single trace of its bract. From these connexions the two bud-traces develop. Since bracteoles are absent from the pedicels, the two bud-traces correspond to the principal veins of the anterior lateral pair of sepals. The pedicel is traversed by a cylindrical stele composed of the traces to the sepals united with those of the other floral organs, but before being inserted on the principal axis, the stele of the pedicel resolves itself into the two bud-traces. In spite of the fact that the pedicel and the bract become fused at their bases, their vascular strands remain distinct, and run parallel for some distance before they turn downwards in the stele of the scape. The further development of the flower is outside the scope of the present paper and was not studied.

## II. *CAMPANULA PERSICIFOLIA* L.

If allowance be made for the difference in habitat, the habit of *Campanula persicifolia* L. is not essentially different from that of *Lobelia Dortmanna* L., so that direct comparison of their inflorescences is possible. The *Campanula*, like the *Lobelia*, perennates as a rosette of foliage leaves, the axis of which elongates in summer as the inflorescence. In each, further rosettes are formed in the axils of the upper rosette leaves which continue the growth of the plant. The principal differences in habit are found in the flowering shoot. In the *Lobelia* this is scapigerous, bearing only reduced leaves, whereas in the *Campanula* the flowering axis bears a close sequence of leaves which form a continuous gradient in size from the rosette to the bracts. This difference is only one of degree, but a further distinction is perhaps more important. In the *Lobelia* the flowers are solitary and without bracteoles, whereas those of the *Campanula* possess bracteoles and frequently flowers form and come to maturity

in their axils. The inflorescence is therefore branched. A difference in the order of maturation of the flowers will presently be described.

Plate IV, Fig. 8, represents a longitudinal section through the apex of a plant in which the buds of the flowers are first appearing. The bracts are formed in precisely the same way as the foliage leaves, from which they cannot be divided by any hard-and-fast line. The internodes remain very short, as during the formation of rosette leaves, a feature which contrasts with the form of the apex in *Lobelia* at a similar stage (Pl. IV, Fig. 5) in which the internodes are greatly elongated. Buds are present in the axils of the bracts which have been cut approximately medianly (Pl. IV, Fig. 8,  $b_1$ ,  $b_2$ , and  $b_3$ ), and it is evident that these buds are being formed, together with their bracts, in acropetal succession.

The further development of the floral buds does not follow the acropetal sequence of their initiation, nor does it proceed in a basipetal sequence such as was described for *Hieracium* in an earlier paper (Philipson, 1948). Pl. IV, Fig. 9, shows that a terminal flower ( $f_1$ ) takes precedence over the flower-buds in the axils of the bracts immediately below it ( $b_2$ ,  $b_3$ ). The dominance of the terminal flower is limited, however, and a bud farther down the axis ( $b_4$ ) can be seen to be equally advanced. A fifth bud ( $b_5$ ) is cut by this section; it is even lower on the inflorescence axis, but can be seen to be in an early stage of development. The sequence of development indicated by this section is maintained until the time of flowering. The actual sequence of anthesis is variable, but the terminal flower opens either first or at the same time as a lateral bloom several nodes below it. Anthesis then proceeds up the stem from this lateral flower and further lateral buds below it frequently develop in a basipetal sequence. Pl. IV, Fig. 10, illustrates the great difference in the degree of development of the terminal and sub-terminal flowers which becomes apparent at an early stage in the development of the inflorescence as a whole.

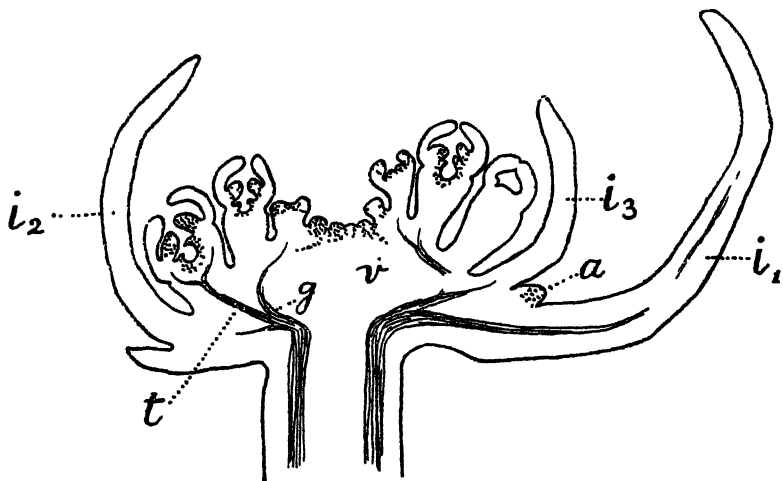
The sequence just described refers to the flowers which terminate the axillary branches. The rudiments of these branches, when they first appear as pockets of meristematic tissue in the axils of the bracts, retain meristematic connexions with the provascular meristem of the stele of the main axis (Pl. IV, Fig. 11). From these connexions the traces of the bracteoles develop. Axillary meristems are present in the axils of these bracteoles (Pl. IV, Fig. 12), and in vigorous plants they develop into flowers which mature after the sequence of primary flowers has been completed.

### III. *JASIONE MONTANA* L.

The flowers of *Jasione montana* L. are arranged in discrete capitate clusters, each group of flowers being surrounded by an involucre of bracts arranged in two series. The flowers are pedicellate—so that the inflorescence is perhaps more correctly referred to as an umbel than a capitulum—and are unaccompanied by bracts, except that the outer flowers lie in the axils of the involucre bracts. It is of interest that the flowers subtended by the involucre

bracts are sometimes aborted (Text-fig. 2), perhaps indicating a tendency towards the involucre with budless bracts which is found in the Compositae.

The development of this inflorescence presents no unusual features. The formation of the involucral bracts and the relation of these with the flowers in their axils is similar to that found in other types. That is to say, the two traces of the flower-buds (there being no bracteoles these correspond to the anterior lateral sepals) are inserted on the sides of the gap corresponding to the single trace of the bract (Text-fig. 2), and the origin of these structures is



TEXT-FIG. 2. Camera-lucida drawing of a longitudinal section through a young inflorescence of *Jasione montana* L. The principal meristematic areas are stippled.  $i_1$ , one of the outer bracts of the involucre;  $i_2$ ,  $i_3$ , inner bracts of the involucre;  $a$ , aborted flower-bud in the axil of  $i_1$  (the bract is cut slightly to one side so that the single median vascular bundle is cut only at its insertion on the stele of the peduncle);  $g$ , border of the gap corresponding to  $i_2$ ;  $v$ , origin of provascular meristem.

essentially similar to that of *Lobelia* and *Campanula*. The flower-primordia arise in strictly acropetal sequence from a meristematic mantle which clothes the somewhat concave receptacle. The floral traces arise as in *Bellis*, from a provascular meristem which becomes separated from the mantle by the maturation of the cortex (Text-fig. 2). The growth of the receptacle is also similar to that of *Bellis*.

The interest of this type lies not so much in details of development as in the formation of a very circumscribed involucre of bracts and the subsequent complete suppression of bracts on the receptacle, on which the flowers arise and come to maturity in a strictly acropetal sequence.

#### IV. DISCUSSION

In the present series of papers descriptions have been given of the development of the inflorescence in species belonging to the Compositae, the Dipsacaceae, the Valerianaceae, and the Campanulaceae. It may be of interest to

compare the development in the types investigated. A comparison has already been made between *Bellis* on the one hand and *Succisa* and *Dipsacus* on the other (Philipson, 1947), and the additional Composite types investigated later (Philipson, 1948) confirmed the view that the capitulum of the Compositae is not strictly comparable with that of the Dipsacaceae. A very similar type of inflorescence is found in *Jasione*, although the pedicellate condition of its florets (a condition not unknown in the Compositae) may justify its formal description as an umbel. Of the two characters which separate the capitula of the Compositae and the Dipsacaceae—namely, the absence of florets in the axils of the involucre bracts of the former and the presence of an outer calyx in the latter—only one separates *Jasione* from the Compositae. That is to say, *Jasione* and the Compositae agree in having no outer calyx or any other trace of bracteoles below their florets, but differ in the presence of florets in the axils of the involucre bracts of *Jasione*. As we have seen, however, these florets may become aborted, when the involucre becomes 'sterile' as in the Compositae, a character of which no trace was found in the Dipsacaceae. To conclude the comparison of these types it may be said that the capitula of neither the Compositae nor the Dipsacaceae approach each other more closely than is permitted by their very general resemblance, whereas the inflorescence of *Jasione* approaches very closely to that of the Compositae.

The non-capitate inflorescences investigated appear to afford a wider basis for these contrasts and resemblances. *Valeriana* possesses an inflorescence in which copiously branched secondary axes spring from each node of the main axis. Inflorescences of similar type are to be found in the Campanulaceae, as in *Trachelium*, in which the bracts of the main axis subtend branches which become subdivided repeatedly. In each case this type of inflorescence appears to be linked with dominance of the terminal flower and a basipetal sequence of flower development. It is as though repeated determination of each axis forced further growth to take place by means of buds in the axils of the bracts and bracteoles.

In *Campanula persicifolia* L. the lateral branches become subdivided only to a limited degree. Correlated with this is the imperfect dominance of the terminal flower, which, as has been described in the present paper, reaches maturity scarcely, if at all, earlier than some lateral flowers, above which the flowers mature in an acropetal sequence. Both these characters are further emphasized in *Lobelia Dortmanna*, for in that species branching of the lateral axes is completely suppressed—or occurs only abnormally and then in imperfect form—and the apex of the main axis is indeterminate. In contrast with this, in the Dipsacaceous genus *Morina*, although the growth of the main axis is indeterminate, nevertheless the lateral axes are branched in a cymose manner (Doll, 1927).

If the *Lobelia* type of inflorescence were to be shortened into a capitate inflorescence a flower-group of the same nature as that found in *Jasione* would result. If the *Morina* type of inflorescence were to be shortened a capitate inflorescence similar to that of *Lonicera* would be produced. (The flowers of

*Lonicera Periclymenum* L. are arranged in decussate dichasia, each group of three flowers being in the axil of a bract on the main axis.)

The Composite capitulum may be derived from an inflorescence of the Jasion type by further suppression of the florets in the axils of the involucre bracts, and the Dipsacaceous capitulum may be derived from the *Lonicera* type by suppression of the lateral florets of the dichasia. This interpretation of the Dipsacaceous capitulum is corroborated by the presence beneath each floret of a whorl of bracts—the outer calyx—and the well-developed medulated stele which supplies each floret in the Dipsacaceae may also indicate the 'branch' nature of the Dipsacaceous floret in distinction to the Composite floret which receives a single solid vascular bundle. It may also be significant that proliferated heads of *Succisa* have been described with dichasia replacing the normal single flowers (Maudslay, 1946).

#### SUMMARY

The development of the hydathode present at the apex of the leaves, bracts, and sepals of *Lobelia Dortmanna* L. is described, and it is concluded that these organs reach a state of functional maturity at a very early stage in leaf development.

The flowers of *Lobelia Dortmanna* L. are arranged in a simple raceme, the flowers developing to maturity in the same sequence as their initiation. Certain buds below the flowers form rudimentary branches but do not normally complete their development. The onset of flowering is first disclosed by an abrupt increase in the length of the internodes, together with a suppression of adventitious roots. The bracts continue the phyllotaxis of the rosette and their initiation and development resembles that of the foliage leaves. As each bract-primordium is formed the portion of the apical meristem in its axil is isolated by vacuolation of the surrounding cells and enlarges to form a floral primordium.

The sequence of development of the flower-buds of *Campanula persicifolia* L. is neither basipetal nor acropetal. The terminal flower develops at a quicker rate than those immediately below it, but the earlier initiated flowers lower on the axis mature in acropetal sequence.

The flowers of *Jasione montana* L. are arranged on a naked receptacle, bracts being confined to the involucre. The flowers in the axils of the involucre bracts are often aborted, a feature which may link this type with the Compositae.

The development of the inflorescences investigated in the present series of papers is analysed, and it is concluded that whereas *Jasione* approaches the Composite type of capitulum, the Dipsacaceous capitulum does not. A comparison of related types suggests that the Dipsacaceous capitulum may be derived from a compacted thyrses rather than a compacted raceme.

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## DESCRIPTION OF PLATE IV

Illustrating Dr. W. R. Philipson's article 'Studies in the Development of the Inflorescence. V. The Raceme of *Lobelia Dortmanna* L., and other Campanulaceous Inflorescences.'

Figs. 1-7. *Lobelia Dortmanna* L.

Fig. 1. Longitudinal section through apex of rosette during production of foliage leaves.  $I_1$ , initiation of leaf-primordium;  $I_2$ , leaf-rudiment, with median provascular meristem;  $I_3$ , slightly older leaf-rudiment with terminal hydathode. ( $\times 75$ .)

Fig. 2. More highly magnified section through Hydathode of leaf of approximately the same age as  $I_3$  in previous figure. The epithema consists of irregularly lobed cells with dense contents. The epidermis is pierced by a stoma at (s). ( $\times 370$ .)

Fig. 3. Longitudinal section through apex of a bract, showing terminal and one lateral hydathode, and the ultimate elements of the vascular system. Note that the epithema cells are scarcely larger than those of the very young leaf ( $I_3$ ) in Fig. 1. ( $\times 75$ .)

Fig. 4. Longitudinal section through rosette, showing (b) bud in axil of upper foliage leaf which would form new rosette during late summer. r, adventitious roots forming close behind stem apex. ( $\times 22$ .)

Fig. 5. Longitudinal section through apex of old raceme. The section passes through the insertion of four bracts on the rachis of the raceme. The flower-rudiments in the axils of these bracts are more advanced in their development in an acropetal sequence. No residue of the apex has remained after the formation of the youngest flower. ( $\times 38$ .)

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Fig 6 Longitudinal section through young scape The bud in the axil of the uppermost rosette leaf is visible at the base of the photograph The internodes between the bracts are elongated close behind the apex ( $\times 16$ )

Fig 7 Longitudinal section through apex of raceme during the formation of a flower-primordium *b* bract with median provascular meristem *f* primordium of flower which is isolated by vacuolated cells from the bract-trace and also from the apical meristem (*m*) ( $\times 220$ )

Figs 8-12 *Canpanula persicifolia* L

Fig 8 Longitudinal section through apex below which the first flower-primordia are forming *b*<sub>1</sub> *b*<sub>2</sub>, *b*<sub>3</sub>, successively older flower-primordia ( $\times 16$ )

Fig 9 Longitudinal section through apex of older inflorescence Note greater development of internodes and advanced condition of the terminal flower-primordium (*f*<sub>1</sub>) and one of the lower buds (*b*<sub>4</sub>) *b* *b*<sub>3</sub>, and *b*<sub>2</sub> are at earlier stages of development ( $\times 16$ )

Fig 10 Longitudinal section through advanced terminal flower and of the flower immediately below it, which has developed more slowly ( $\times 16$ )

Fig 11 Tangential longitudinal section through inflorescence passing through primordium of lateral flowering branch (*b*) and the leaf (*l*) in the axil of which it lies *v* provascular meristem connecting primordium to stele of main axis of inflorescence ( $\times 60$ )

Fig 12 Tangential longitudinal section through older lateral flowering branch *f*<sub>1</sub>, flower terminating branch, *br*, bracteole of terminal flowers, *f*<sub>2</sub>, primordium of flower forming in the axil of the bracteoles ( $\times 60$ )



PHILIPSON —Studies in the Development of the Inflorescence V



# Experimental and Analytical Studies of Pteridophytes

## XII. The Effect of Different Concentrations of Oxygen on Inactive and Active Meristems of Ferns

BY

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With sixteen Figures in the Text

### INTRODUCTION

COMPARATIVELY little is known of the physiological factors which control the inception of form and structure in the undifferentiated meristematic tissue at the growing-point. White (1939) has produced evidence that the partial pressure of oxygen may be one of the factors which control differentiation in a plant-tissue culture: undifferentiated cultures of a *Nicotiana* callus, growing on the surface of a semi-solid medium, were observed to become differentiated and to produce leafy branches on being immersed under 8 mm. of a liquid culture medium; roots were not obtained. The suggestion that the reduced oxygen supply may be a factor in controlling morphogenetic processes is important and deserves further consideration. In the present paper we have undertaken a more extended investigation of the effect of different partial pressures of oxygen on the growth and morphology of inactive and active meristems in three different ferns.

During the progress of this work Levine (1947) has published data which indicate that a reduced oxygen supply is not a necessary condition for differentiation in a plant-tissue culture. In several instances tissue masses of carrot-root meristem cultures produced complete morphological differentiation of the tissue into root stem and leaf. The precise conditions determining this differentiation were not established, but there was no suggestion that altered oxygen gradients were involved.

### MATERIALS AND METHODS

The development of buds in *Matteuccia struthiopteris* Tod., *Onoclea sensibilis*, and *Dryopteris aristata* has been described in considerable detail by Wardlaw (1943, 1943a, 1946). It was evident from this work that the rhizomes of *Onoclea* and *Matteuccia* would provide favourable materials for the present investigation. In each, buds are normally absent but appear at definite points on the rhizome when the apical meristem is damaged or

removed. The induced plantlings arise superficially in proximity to points of conjunction of two meristemes of the dictyostelic vascular system; at these positions small areas of distinctive meristematic cells—which have been described as detached meristemes or bud rudiments—are present in a quiescent condition in the normal rhizome (Wardlaw, 1943). A short length of rhizome, 4–5 cm., may possess 3–5 detached meristemes. If, therefore, segments of rhizomes are placed in vessels supplied with different concentrations (or partial pressures) of oxygen (in nitrogen) under conditions suitable for further growth, the detached meristemes will be exposed to the different atmospheres from the outset, and any growth or morphogenetic activity will have taken place under uniform controlled conditions. In particular, the nature of the gaseous environment will be precisely known.

The rhizomes of *Onoclea* and *Matteuccia* were prepared for use by removing all soil, roots, and leaf bases. They were then cut into segments about 4 cm. long, several detached meristemes being available for development in each piece. Four pieces from each species were at once placed in each of the growth chambers of the apparatus. The remainder were planted in peat and kept in a warm greenhouse to induce development of the meristemes, in preparation for the second part of the investigation.

Shoots of *Dryopteris aristata* were collected in the open in spring while still dormant and were prepared for the growth chambers by having all roots and old leaf bases trimmed off. All but the youngest leaf primordia were also removed, the untreated apical region being easily distinguishable from the adjacent tissue which became brown and corky. Actively growing shoots were collected from the same locality later in the year and were prepared for treatment in the same way.

## RECORDS

The drawings illustrating this account were taken from the best grown plants in each tube. But although they are representative of the maximum growth observed under the particular conditions, other plants from the different tubes would have shown approximately the same degree of difference. Photographic records prepared by Mr. E. Ashby are available for inspection.

## APPARATUS AND PROCEDURE

Clear glass tubes, 1 ft.  $\times$  1.25 in., closed at each end by rubber bungs, were used as growth chambers. For growth in darkness some of the tubes were painted black. The assembled apparatus consisted of the following units in sequence: (a) cylinders with fine adjustment valves, containing compressed gas mixtures; (b) 10 per cent. NaOH solution, to remove carbon dioxide; (c) 0.01 N Ba(OH)<sub>2</sub> solution, tinted with phenolphthalein, to indicate the complete removal of carbon dioxide and to saturate the gas mixture with water vapour; (d) forked tube to supply the gas mixture to (e) a dark chamber and (e') a clear chamber, the rate of flow being adjusted by screw clips on the stout connecting rubber tubing; the growth chambers were immersed in

water in a glass tank maintained at 20° C. by thermostatic control; (f) manometer with Brodie fluid at the outlet of each growth chamber; (g) gas sampling tube; and (h) calibrated bubbler containing water, which sealed off the system from direct contact with the air and provided a measure of the rate of gas flow.

The flow of gas through the apparatus was adjusted to approximately 250 c.c. an hour. After initial tests it was found sufficient if the contents of the gas sampling tubes were analysed in a small Haldane apparatus at approximately fortnightly intervals. During the course of the experiment there were no appreciable changes in the supplied gas mixtures. The carbon dioxide produced by the respiration of the plant material fluctuated between 0.1 and 0.4 per cent. This concentration is rather higher than would have been desired, but as shown by the review of Clements (1921) and later work, much higher concentrations are required to cause injury in most mesophytes. No special steps were taken to reduce the observed concentrations.

Four different gas mixtures were supplied to the growth chambers: (1) 44.6; (2) 20.9; (3) 11.4; (4) 6.19 per cent. oxygen in nitrogen.

At the beginning of the experiment the temperature was maintained at 20°, but later in the year the external temperature exceeded this figure. Apparatus was not available to reduce the temperature of the bath to its original value.

#### INACTIVE AND ACTIVE MATERIALS

At the beginning of the experiment (April 3, 1947) the materials placed in the tubes were in a quiescent condition. These were removed for examination at suitable intervals and samples fixed for sectioning. A second lot, i.e. those which had been planted in peat in the greenhouse at the beginning of the experiment, were introduced into the growth tubes on May 27, 1947. These now showed evidence of growth: the *Matteuccia* segments had produced small hemispherical masses of tissue from the meristematic areas, while the *Onoclea* pieces had formed small green plantlings ranging in length between 0.4 and 0.7 cm. The second set of *Dryopteris* apices were also in an active state of growth when placed in the growth tubes on June 24, 1947.

#### EXPERIMENTAL OBSERVATIONS

##### (a) *Effect on dormant meristems*

*Dryopteris*. In the *light*, growth took place in all the gas mixtures, and green leaves of normal appearance were developed. No great differences in the rate of production and growth of leaf primordia were noticed in the different gas mixtures. In this respect *Dryopteris* is very different from *Onoclea* and *Matteuccia*. As had been anticipated, this material is less suitable than *Matteuccia* and *Onoclea* for observation of the effects of the different oxygen concentrations on the general morphology, and it remains to be seen whether or not the microscopical examination of the apical region will yield points of interest.

In the *dark*, growth occurred in all four oxygen concentrations but was soon interrupted in the 6 per cent. oxygen by fungal attack and subsequent rotting of the material. Two separate replacements suffered similar attacks. In the remaining mixtures the leaf primordia produced etiolated growths each consisting of a pale rachis, stouter than normal, with an in-rolled tip but no lamina. The appearance was similar in each mixture. Growth was less in the 11 per cent. oxygen, but there were no evident differences in the other two mixtures.

*Onoclea and Matteuccia.* The materials in each tube developed with considerable uniformity. Well-marked differences between the two species and between the materials of each species in the different gas mixtures were noted. The relevant data are set out in the accompanying table.

The first signs of growth were noted in the clear tubes after 11 days. The detached meristems of *Onoclea*, which at the outset were scarcely discernible, were now conspicuous in all the oxygen mixtures as bright green, slightly raised cushions of tissue, but in *Matteuccia* this appearance was only evident in the 45 per cent. oxygen.

As an indication of the kind of observations that were made, the state of affairs on the 34th day of the experiment may be briefly summarized.

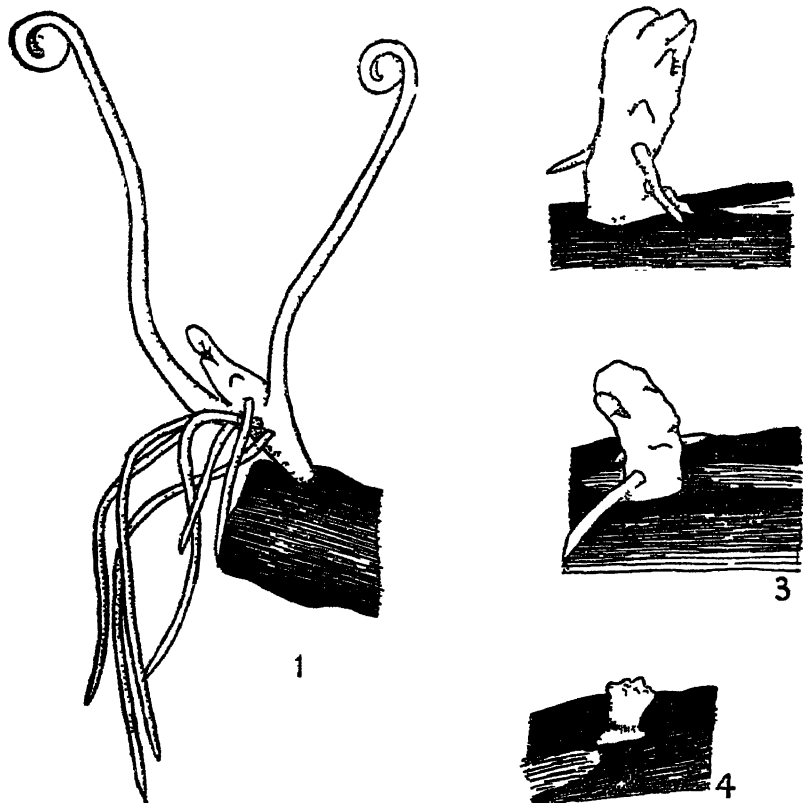
In the *light*, in 45 per cent. oxygen, the *Onoclea* rhizome had produced conspicuous plantlings with several leaves and roots; in the 21 per cent. oxygen the plantlings were small, without clearly defined leaves and with a few small roots; in 11 per cent. oxygen the plantlings were appreciably smaller; while in 6 per cent. growth was very slight, only the first signs of an apical region being perceptible. In *Matteuccia* growth was much slower. In 45 per cent. oxygen no leaves had been formed, maximum growth being shown by a small cylindrical plantling with a simple apical region and a small root; in 21 per cent. oxygen the bulging meristems were just developing apical zones; in 11 per cent. the meristems were small green bulges; and in 6 per cent. the detached meristems did not project above the surface of the rhizome but were of a bright green colour (Figs. 1-8).

In the *dark*, in both *Onoclea* and *Matteuccia*, considerably more growth had taken place, but the general relationships of growth described above were maintained in the different gas mixtures (Figs. 9-16). In *Onoclea* large plantlings were found in 45 and 21 per cent. oxygen; in 11 per cent. the plantlings were smaller, but, unlike those in the light, had actively developing young leaves; in 6 per cent. oxygen no leaf rudiments were visible. In 45 per cent. oxygen the *Matteuccia* plantlings had well-developed young leaves; in 21 per cent. the leaf primordia were small and several roots were developing; in 11 per cent. the detached meristems had produced only small outgrowths of pale tissue; while in 6 per cent. oxygen only a slight swelling of the meristematic area was discernible.

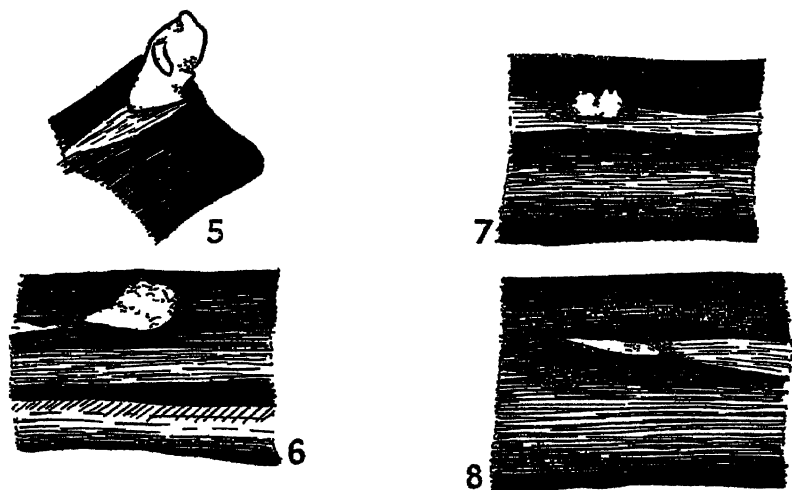
No particular features in the external morphology of any of the plantlings call for comment. It will be noted that as the experiment progressed the size differences of the plantlings in different oxygen concentrations was greatly

TABLE I  
Growth of Initially Inactive Fern Meristems in Different Concentrations of Oxygen  
ONOCLEA SENSIBILIS

Percentage concentration of oxygen.	DURATION OF EXPERIMENT (DAYS)		
	11 days.	34 days.	43 days.
<i>Light.</i> 6			
11	Green meristematic cushions.	Green cushions. Very small plantings. Small plantings: no leaves, few small roots. Conspicuous plantings: several leaves and roots.	Green cushions or small leafless plantings. Small plantings: few leaves. Medium-sized plantings. Strong plantings: many leaves and roots.
21			
45			
<i>Dark.</i> 6	No examination.	Small leafless plantings. Small plantings with developing leaves. Large plantings with uncoiling leaves. Large plantings with uncoiling leaves.	No appreciable change. Etiolated plantings larger than those in light; plantings in 11% much smaller than in 21% and 45%.
11			
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<i>MATTEUCCIA STRUTHIOPTERIS</i>			
<i>Light.</i> 6			
11	No visible development.	Small green meristematic areas. Green meristematic cushions. Cushions beginning to bulge. Small cylindrical plantings: no leaves or roots.	Low meristematic cushions. Apical region developed on cushions. Small plantings with roots and roots.
21			
45			
<i>Dark.</i> 6	No examination.	Pale green meristematic areas. Small pale green cushions. Small plantings with leaf primordia and roots. Large plantings with well-developed leaves and roots.	Little further development. Larger than on 34th day; plantings in 45% O <sub>2</sub> larger than in 21%.
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<i>Dark.</i> 6	No examination.	Pale green meristematic areas. Small pale green cushions. Small plantings with leaf primordia and roots. Large plantings with well-developed leaves and roots.	Little further development. Larger than on 34th day; plantings in 45% O <sub>2</sub> larger than in 21%.
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<i>MATTEUCCIA STRUTHIOPTERIS</i>			
<i>Light.</i> 6			
11	No visible development.	Small green meristematic areas. Green meristematic cushions. Cushions beginning to bulge. Small cylindrical plantings: no leaves or roots.	Low meristematic cushions. Apical region developed on cushions. Small plantings with roots and roots.
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<i>Dark.</i> 6	No examination.	Pale green meristematic areas. Small pale green cushions. Small plantings with leaf primordia and roots. Large plantings with well-developed leaves and roots.	Little further development. Larger than on 34th day; plantings in 45% O <sub>2</sub> larger than in 21%.
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<i>MATTEUCCIA STRUTHIOPTERIS</i>			
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<i>Light.</i> 6			
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<i>MATTEUCCIA STRUTHIOPTERIS</i>			
<i>Light.</i> 6			
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<i>MATTEUCCIA STRUTHIOPTERIS</i>			
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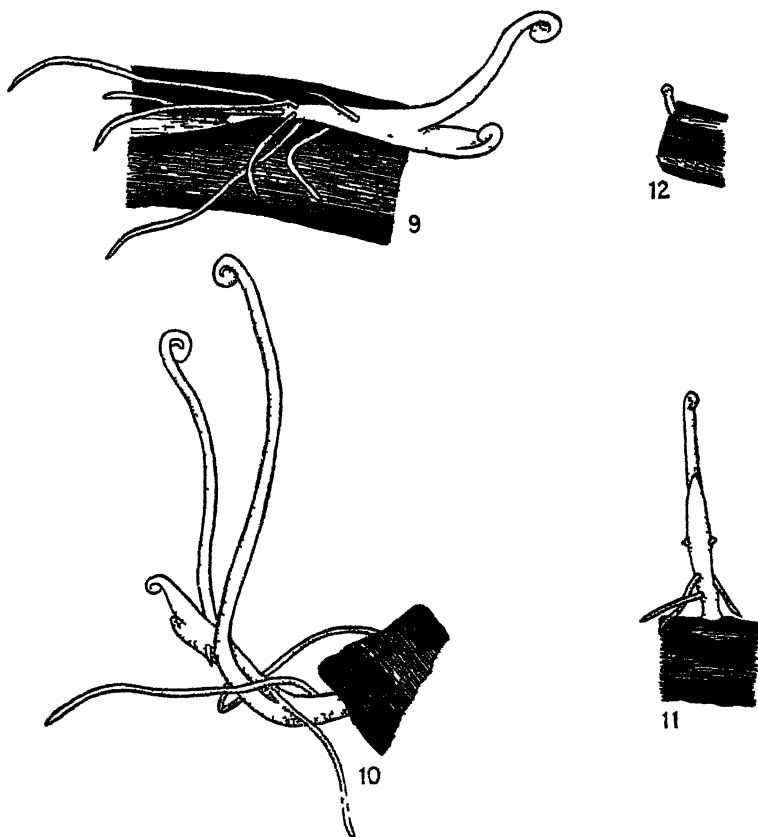
FIGS 1-4. *Onoclea sensibilis*. Growth after 34 days in light from 'detached meristems' of rhizomes supplied with 45, 21, 11, and 6 per cent oxygen respectively (Fig 1  $\times 23$ ; Figs 2-4  $\times 46$ )



FIGS 5-8. *Matteuccia struthiopteris*. Growth after 34 days in light from 'detached meristems' of rhizomes supplied with 45, 21, 11, and 6 per cent oxygen respectively. ( $\times 46$ )

reduced, probably as a result of the inception of active photosynthesis in the expanded leaves. In 6 per cent. oxygen the plantlings of *Onoclea* in the light remained small until late in the experiment.

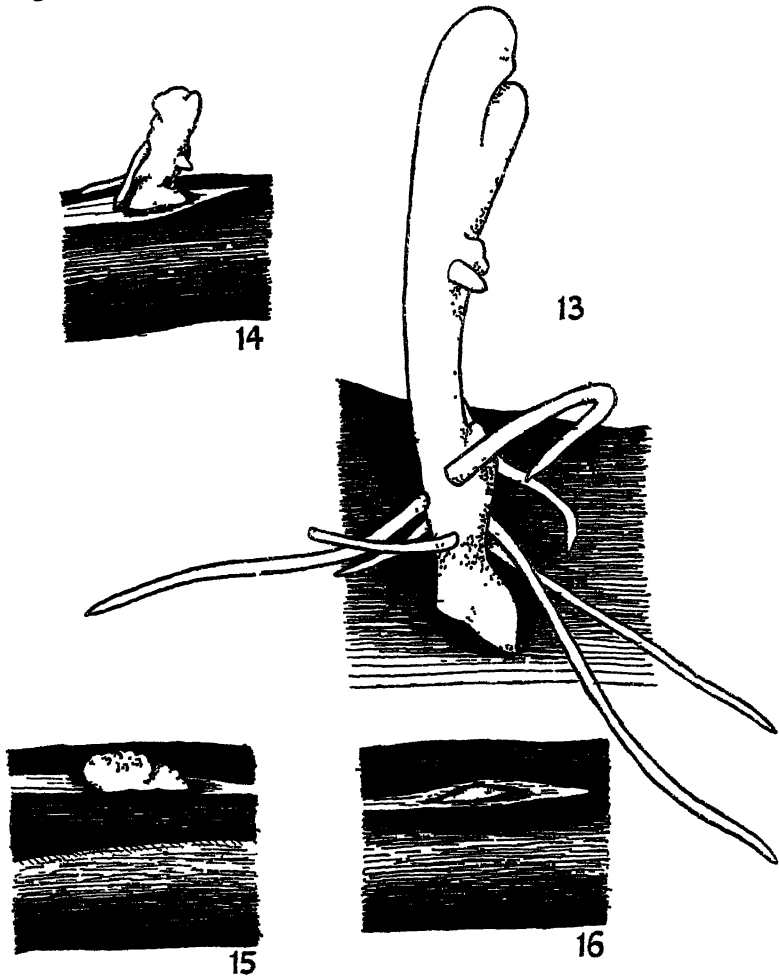
*Onoclea* plantlings in the dark, after 83 days, revealed marked differences from those in the light. The former were all appreciably larger than the



FIGS 9-12 *Onoclea sensibilis* Growth after 34 days in dark from 'detached meristems' of rhizomes supplied with 45, 21, 11, and 6 per cent oxygen respectively ( $\times 1.75$ )

latter and the size differences in the different oxygen concentrations were still clearly maintained. The plantlings in the dark tubes were strikingly etiolated. The long, slender, colourless rhizomes gave rise to numerous long leaf-stalks with circinate tips, but in no plantling was there any expansion of the lamina. Many leaf-stalks in the three higher oxygen concentrations exceeded 15 cm. in length. One in 45 per cent. oxygen had a total length of 16.5 cm. and was forked 3.5 cm. from the distal end. In 6 per cent. oxygen the plantlings were small and attenuated, but larger than those in the light. They were still very small on the 90th day of the experiment, but after 130 days they were quite large and showed no evident differences from those observed earlier in the higher oxygen concentrations.

*Matteuccia* plantlings in the *light* grew consistently more slowly than those of *Onoclea*. In 45, 21, and 11 per cent. oxygen, however, after 90 days, the plantlings were well grown and bore numerous green leaves, i.e. with the



FIGS. 13-16. *Matteuccia struthopters*. Growth after 34 days in dark from 'detached meristems' of rhizomes supplied with 45, 21, 11, and 6 per cent. oxygen respectively. ( $\times 46$ )

onset of active photosynthesis the earlier differences in growth-rate had practically disappeared. In some instances more than one growing-point had been developed from the original meristematic area but, as with similar developments in *Onoclea*, this could not be correlated with oxygen supply. By the 132nd day, several small plantlings had appeared in the 6 per cent. oxygen: apart from their size these showed no unusual features in their external morphology.

All *Matteuccia* plantlings produced in the dark were characteristically

etiolated, their elongated stems being in striking contrast to the short stocks produced in the light. No laminae were developed on the long leaf-stalks; in the larger leaves the pinnae were represented by small, widely separated outgrowths of the rachis. Small amounts of chlorophyll were present.

(b) *Effect on active meristems*

*Dryopteris*. Actively growing shoots, trimmed as described, were placed in the growth tubes on June 24. After 50 days the shoots in 6 per cent. oxygen in both light and dark had been destroyed by fungal invasion. The others were all developing, but growth had been slow and no evident differences in the external morphology were observed. Materials were fixed for sectioning.

*Onoclea and Matteuccia*. Two pieces of rhizome of each species were placed in each tube on May 27. By this time the *Onoclea* had produced small cylindrical plantlings without leaves ranging from 0.4 to 0.7 cm. in length; the *Matteuccia* had only formed small green outgrowths of meristematic tissue. The behaviour of both species was remarkably uniform in the several concentrations of oxygen and strikingly different from that of the material introduced in the dormant condition. Thus in the *Onoclea* material after 28 days in the *light*, closely comparable amounts of growth had taken place at all the oxygen concentrations. No differences in external morphology were observed, nor did the plantlings differ from those which had developed from the dormant condition.

In the *dark*, development in *Onoclea* was also approximately equal in all four oxygen concentrations. The initially green plantlings became etiolated. The rhizomes had an evident tendency to grow erect. This effect had also been noticed in the earlier batch in the dark. Expanded laminae were not formed.

When the *Matteuccia* materials were examined after 35 days in the *light*, approximately equal development had been reached in 45, 21, and 11 per cent. oxygen and no differences in external morphology were observed. In 6 per cent. oxygen the plantlings were appreciably smaller, but they were still far in advance of those which had developed in this concentration from the initial dormant condition. In the *dark*, during the same period, comparable amounts of growth were observed in 45, 21, and 11 per cent. oxygen. In 6 per cent. oxygen, only small plantlings had developed. In the course of 77 days these had attained a considerable size.

#### GROWTH IN OXYGEN AND IN NITROGEN

In a further series of experiments, the growth of inactive meristems of *Onoclea* and *Matteuccia* in pure oxygen and pure nitrogen was studied, comparisons being made with similar lots of material in 21 per cent. oxygen and 6 per cent. oxygen after 34 days. In the *light*, well-developed plantlings were produced by the *Onoclea* rhizomes in 21 per cent. oxygen; in 6 per cent. oxygen growth was very slight; in nitrogen there was no evidence of growth and the tissues decayed; in oxygen there was no growth, and several pieces of

rhizome succumbed to fungal invasion. In the *light*, the *Matteuccia* rhizomes produced green bulges of tissue in 21 per cent. oxygen, but in 5 per cent. the detached meristems, although evidently active, showed no projection from the rhizome; in nitrogen the tissues decayed without any indication of growth, while in oxygen bulging of the meristems was evident.

In the *dark*, both *Onoclea* and *Matteuccia* rhizomes decayed in nitrogen; in 6 per cent. and 21 per cent. oxygen no unusual developments were noticed. *Onoclea* rhizomes in oxygen developed large bulges of meristematic tissue quite different in appearance from the narrower projections developed in normal concentrations. In the *Matteuccia* rhizomes similar bulges were forming, but these were less well developed than those of *Onoclea*.

### DISCUSSION

Definite evidence has been obtained for three ferns that the external morphology of plantlings is not affected when they are grown from inactive meristems in different concentrations of oxygen. This holds for growth in the dark as well as in the light. The same conclusion was reached in experiments in which active meristems were grown in different concentrations of oxygen. These observations therefore give no support to the general hypothesis that the oxygen concentration is either a specific or a direct morphogenetic factor in the development of meristematic tissue. In particular, the data so far obtained do not support the suggestion of White (1939) that a diminished or limited supply of oxygen may be a factor in the differentiation of organs and tissues. It still remains to be ascertained what changes, if any, have been produced internally as a result of exposing the growing meristems to different concentrations of oxygen. This will be the subject of a later paper.

Although the external morphology of plantlings was unaffected, their growth rates from originally inactive meristems in the different concentrations of oxygen were strikingly different. In *Onoclea* and *Matteuccia* there was a marked increase of growth, both in the dark and in the light, with increasing concentrations of oxygen. Growth was absent in pure nitrogen and very slow in 6 per cent. oxygen; but in 11 per cent. growth was not greatly different from that in normal atmosphere. In pure oxygen growth was slow and, as the preliminary data indicate, probably of an abnormal character. The results with *Dryopteris* were less clearly defined; in general this material appeared to be less influenced by the oxygen concentration. A difference in response to oxygen concentration has also been noticed in the growth of prothalli of *Dryopteris* and *Onoclea* (Allsopp, unpublished results). The gradation in growth with oxygen concentration observed in *Onoclea* and *Matteuccia* is comparable with that in many spermatophytes, as is evident from the monograph of Clements (1921).

The considerable differences in the growth-rates of plantlings from initially inactive meristems in the several oxygen concentrations was not observed in materials, in which the meristems were already active, when similarly exposed to different atmospheres. The explanation of this difference between inactive

and active meristems probably lies in the dependence of the plantlings during the early stages of growth on the food reserves of the rhizomes. In an active rhizome the reserves will have been partially mobilized, and the materials required for the growth of plantlings will already be available: in an inactive rhizome the formation of such growth materials is apparently enhanced by a higher oxygen concentration and retarded by a lower. An explanation along these lines is supported by the observation that as plantlings acquire actively photosynthesizing leaves they become less dependent on the food reserves in the rhizome and the size differences in the several oxygen concentrations are greatly reduced.

Materials grown in the dark show that etiolation is not influenced by different oxygen concentrations. Our results extend the observations of Hommer (1926) on etiolation in ferns to a few additional species: we agree with him that the general appearance is similar to that of etiolated spermatophytes. The presence of small amounts of chlorophyll is usual for etiolated ferns. Neither *Onoclea* nor *Matteuccia* developed expanded laminae. One of the most striking effects was the production of long rhizomatous shoots in *Matteuccia* in contrast to the short, stout, erect shoots produced in the light. Wardlaw (unpublished results) had already found that when pieces of *Matteuccia* rhizome were buried in a foot of soil long shoots were obtained from the meristematic areas, and rose to the surface before producing normal leaves. The present work indicates that this effect is directly connected with growth in darkness and is not a result of diminished oxygen supply.

#### SUMMARY

Rhizomes of *Onoclea sensibilis* and *Matteuccia struthiopteris*, with detached meristems or bud rudiments (*a*) in the inactive state and (*b*) in an active condition, and shoot apices of *Dryopteris aristata*, were placed in oxygen-nitrogen mixtures containing 45, 21, 11, and 6 per cent. oxygen, and in pure oxygen and pure nitrogen in light and in darkness.

All materials, except those in nitrogen, eventually showed evidence of growth. No differences were observed in the external morphology of the new growths as a result of exposure to the different concentrations of oxygen.

In *Onoclea* and *Matteuccia* the rate of development of plantlings from initially inactive meristems increased with the concentration of oxygen up to 45 per cent.; growth in pure oxygen was slow and probably of an abnormal character. This relationship was not evident in *Dryopteris*. The growth of plantlings from initially active meristems was not appreciably different in the various mixtures.

The results obtained, which are discussed in relation to other work, do not support the hypothesis that the concentration of oxygen is a direct or specific morphogenetic factor in the development of fern meristems.

An explanation of the effect of different concentrations of oxygen on the growth of plantlings is suggested.

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# The Functional Significance of Vascular Anastomoses in determining the Water-supply to Leaves in *Eupatorium adenophorum*<sup>1</sup>

BY

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With nine Figures in the Text

## INTRODUCTION

IT is generally supposed that a free interchange of water can take place between the vascular traces of leaves and the surrounding parenchymatous tissue. This, no doubt, is true, but when the dynamic aspect of water-supply is considered, the question of resistance to this movement assumes importance. The forces of suction pressure determining the direction of movement are great, but the rate of exchange will be conditioned by the resistance offered by the living tissues. Much previous work has shown that the resistance to movement of water through living cells is very high (Baptiste, 1935; De Haan, 1933; Huber and Höfler, 1930) and probably this factor plays a much greater part in water movement than is generally envisaged. Thus, for instance, in the simplest case of water-supply to the lamina of a leaf, the main resistance occurs not in the capillaries of the xylem but at the vessel endings where the water has to traverse the living cells of the leaf. Experiments performed by the writer (1940) have shown that transpiration, under constant conditions, is scarcely affected when the main veins of the leaf of *Pelargonium zonale* are severed; an estimate of the resulting change in suction pressure indicated that this was very small, so that resistance to water movement in the fine veins must be quite low as compared with the total resistance encountered in the passage of water.

Experiments were therefore undertaken to investigate this question more thoroughly. To do this successfully it is necessary to force, if possible, movement of water across living tissue, and this can only be ensured by interrupting the continuous water channels in the xylem. In shoots which have reached the stage of formation of secondary wood there is an uninterrupted connexion between the traces of leaves inserted at different levels, but in the primary shoot the leaf traces are separate and therefore, if water is to pass between leaves inserted at different levels, living parenchyma tissue must be traversed if the supply of water from regions below is blocked. Such experi-

<sup>1</sup> The facts presented were included as part of a thesis submitted to the University of London for the Ph.D. degree in 1941.

ments are reported in this paper using the shoot of *Eupatorium adenophorum* prior to the onset of secondary thickening. This plant was chosen for convenience as it was available in a leafy condition during the winter when work was begun. The details of the vascular anatomy were at that time unknown, and it was only the surprising results of the physiological experiments performed, and the hypotheses advanced to explain these, which made a study of the anatomy imperative. This species of *Eupatorium* has typical mesophytic leaves on long petioles arranged in decussate pairs which are widely separated by internodes.

### EXPERIMENTAL

The material used for these experiments was the primary shoot of the plant consisting of four or fewer internodes with their attendant leaves. The shoots

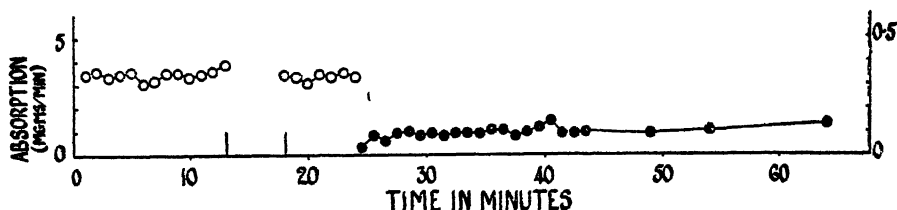


FIG. 1. The absorption of water at the base of the shoot (white points, left-hand scale) and via the petiole (black points, right-hand scale). The two uprights (between 10 and 20) indicate the time during which the petiolar supply was fitted.

were detached and supplied with air-free distilled water from a calibrated potometer. The connexion was made by slipping a short length of valve rubber tubing over the cut end, and inserting this into a tapered glass tube (Gregory, 1938). When desired, water was passed into the plant through a petiole, after removal of the lamina, in the same way, but in this case the adaxial groove was filled with plasticine before the rubber tube was attached. To cut off the water-supply from the base of the shoot, the potometer was removed and the exposed end was embedded in vaseline.

To maintain constant conditions of transpiration the shoot was set up either under a bell jar, kept at constant temperature and humidity, or in a constant-temperature greenhouse. Artificial illumination from a 200-watt water-screened lamp was provided in all experiments. After setting up, the shoot was allowed to remain for 2 hours under the experimental conditions to reach a steady rate of transpiration before readings were begun.

#### *Experiment 1*

A portion of a shoot, bearing two pairs of fully expanded leaves, was removed from the plant; the upper cut end of the shoot was covered with vaseline and the lower end fitted into a calibrated potometer and readings were taken at minute intervals. When the rate of absorption was steady a

second potometer was fitted to the petiole of one of the lower leaves and the absorption of water from the basal and petiolar supplies was now followed. Subsequently the basal supply was blocked by removing the potometer and sealing the exposed end of the stem with vaseline, when observations were made only of the petiolar absorption of water.

The results of two such experiments are shown in Figs. 1 and 2, in which the absorption in mg./min. is plotted against time. The two short vertical marks indicate the time during which the petiolar supply was fitted; the white points (left-hand scale) and black points (right-hand scale) show the basal and petiolar absorption respectively. In Fig. 1, after the interruption of the readings while the petiolar supply was fitted, both potometers were read for a further period of 7 minutes. During this time the uptake through the base continued

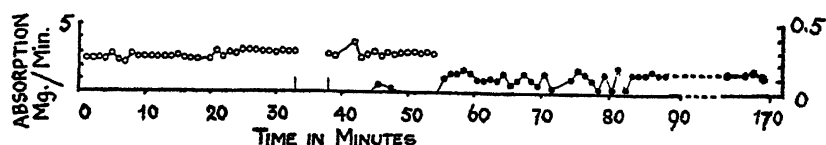


FIG. 2. The absorption of water at the base of the shoot (white points, left-hand scale) and into the petiole (black points, right-hand scale). The two uprights indicate the time during which the petiolar supply was fitted.

as before while no water entered through the petiole. The basal supply was then cut off and water absorption through the petiole began. Having due regard to the difference in scales in the diagram, it will be seen that the petiolar uptake was very small and maintained a maximum rate of 0.1 mg./min. as compared with 3.5 mg./min. through the end of the shoot. In Fig. 2 a similar experiment of much longer duration is shown and provides confirmatory evidence.

It was found that 20–40 minutes after the removal of the basal supply the leaf opposite the supply petiole was distinctly flaccid, while the upper pair of leaves were still turgid. Later, however, all the leaves became flaccid. Fig. 3 shows the condition of the shoot immediately after the removal of the basal supply and after an experimental period of 1 hour.

The conclusions to be drawn from this experiment are as follows:

- (a) There is no free interchange of water between the four leaves on the shoot.
- (b) The resistance to the entry of water through the petiole is much greater than through the cut end of the stem as is shown by the relative absorption rates. Since conditions of transpiration remained constant the rates are a direct measure of the resistance along the two paths.
- (c) The increased resistance to water entry has resulted in a rise in suction pressure of the shoot by loss of water from the leaves, but even when the suction pressure of the leaves has reached its maximum possible value, and wilting has begun, the increased pull is still insufficient materially to increase uptake through the petiole.

*Experiment 2*

The previous experiment showed that direct vascular connexions were unlikely between leaves at the same or at adjacent nodes, and this experiment was therefore carried out to find whether a direct vascular connexion existed between leaves inserted on the same orthostichy and thus two nodes apart.



FIG. 3. The condition of the shoot at the beginning (left-hand) and at the end (right-hand) of an experimental period of 1 hour. The potometer was attached to the lower right-hand petiole shown with the lamina removed. This figure was traced from photographs.

A portion of the shoot, bearing three pairs of fully expanded leaves, was used and treated as in expt. 1; the results are shown in Fig. 4. Making due allowance for the difference in scales in water uptake, the result is similar to that derived from expt. 1. The uptake through the petiole, however, finally reached twice the value of that recorded in the previous experiment. To test whether this higher rate was due to the influence of the leaf inserted above the petiolar supply, this leaf was removed some 45 minutes after the beginning of the experiment at the instant indicated in the diagram by the arrow. It is apparent that the uptake is quite unaffected.

The experiment indicated that the vascular supply of the six leaves is again independent.

It seemed probable from these experiments that no anastomoses of leaf traces occurred through two internodes. The limitations of this method of experimentation now became apparent, for if a region of the shoot long enough to explore possible connexions were used it would have necessitated employing

parts of the shoot in which secondary thickening had begun. The following method was therefore used.

Attachment to a potometer was made at the apical as well as at the basal end of the shoot. The apical bud and immature parts of the shoot were removed leaving a variable number of leaves.

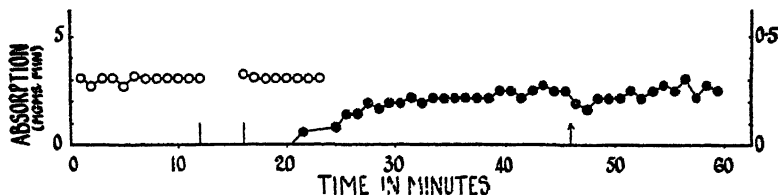


FIG. 4. Basal absorption (white points, left-hand scale) and petiolar absorption (black points, right-hand scale). At the time indicated by the arrow the leaf immediately above the supply petiole, but two nodes distant, was removed.

### Experiment 3

Shoots were prepared bearing one and two pairs of expanded leaves, the internodes above and below being severed midway between the nodes. These shoots were supplied with water at the apical end only, the base having been blocked with vaseline. The shoots were left for a considerable time, after which it was found that all the leaves were wilted. The results therefore of this experiment were precisely similar to those already recorded for shoots supplied through the petiole and the same conclusions may be drawn.

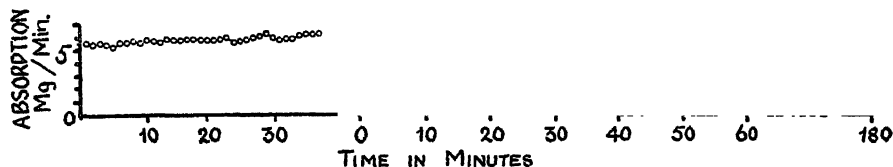


FIG. 5. The basal absorption of water (white points) prior to attachment of the apical supply, and the basal absorption after attachment of the apical supply (black and white points). The black points show the apical absorption. The basal supply was removed 50 min. after the apical supply was fitted.

### Experiment 4

When an experiment similar to expt. 3 was performed with a shoot bearing three pairs of leaves a very different result was obtained. In this preparation water uptake, apically and basally, was measured by attaching potometers. The water entered the shoot from above along the traces of the fourth pair of leaves above the basal pair.

To obviate hydrostatic pressure differences the shoot was set up in a horizontal position. After the usual 2-hour preliminary period, absorption of water was followed through apex and base simultaneously and later through apex alone after blocking the basal supply. The results are shown in Fig. 5. It may be emphasized here that only one scale is necessary in this diagram.

For the first 40 minutes basal absorption was recorded and remained substantially constant. The record is interrupted for the period necessary to

attach the apical potometer and to allow the establishment of the new steady conditions. Readings were now taken of both basal and apical uptake, the black points showing basal and apical uptakes. It is apparent

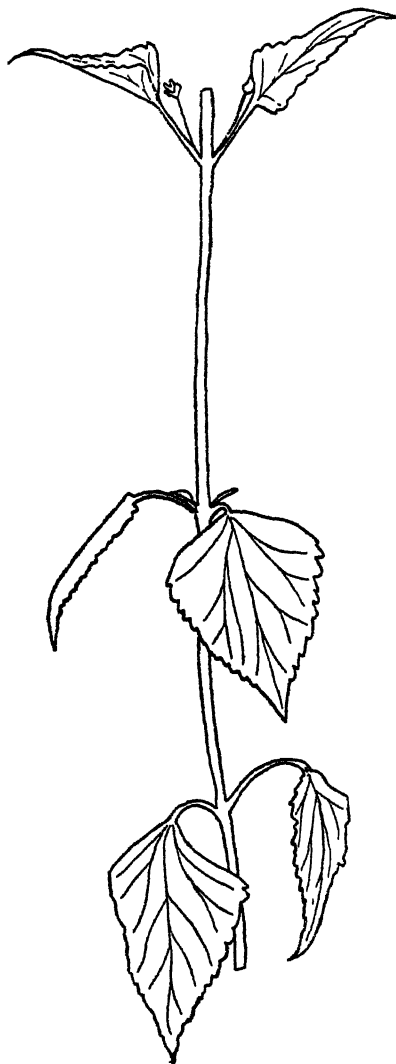


FIG. 6. The condition of the shoot at the end of experiment 4 is shown.

that together the uptake was about equal to that of the previous basal absorption alone, and that the distribution of water more or less reflected the number of leaf traces in the two regions of the shoot. After 50 minutes the basal supply was blocked and absorption through the apex alone continued. Within a few minutes uptake at the apical end had risen to a value equal to that pre-

viously entering the base when both supplies were available, and as the experiment continued the absorption rate steadily rose to a value approaching that previously obtained for basal absorption alone at the beginning of the experiment. Fig. 6 shows the condition of the shoot at the end of three hours. The lowermost leaves are wilted, the intermediate leaves are flaccid, while the uppermost leaves are quite turgid. The suction pressure in the lower leaves has evidently increased considerably and the progress of the rising suction pressure while the water content of the leaf was falling is reflected in the increasing absorption rate already noted in Fig. 6. The rather lower final absorption rate as compared with the original uptake through the base of the shoot no doubt was due to stomatal closure in the basal leaves although this is conjectural, as stomatal behaviour was not followed.

The following conclusions from this experiment may be drawn:

- (a) A direct connexion exists between the leaf traces at the apex of the shoot (i.e. those of the fourth pair of leaves from the base of the experimental shoot) and those of the third pair of leaves from the base. The leaf traces of these two pairs of leaves must anastomose in the region of the lowermost node. This was confirmed by the observation that when the lowermost pair of leaves were removed, by cutting midway through the internode above, the uppermost pair of leaves very soon wilted.
- (b) So long as the connexion between the leaf traces remains unbroken water will pass readily up and down the stem as the high rate of absorption through the apex of the shoot seen in Fig. 5 indicates. As soon as water has to pass across parenchymatous tissue, resistance to its movement increases to such an extent that the maximum suction pressure exerted by the flaccid leaf is insufficient to maintain a flow of greater than one-tenth of the normal rate.

### Experiment 5

Further experiments were now carried out to confirm the previous findings. To do this, the path of the water in the vascular system was followed by the use of a dye solution (2 per cent. naphthal orange). A small evaporimeter cup of unglazed porcelain was attached to the apex of the shoot, the leaves having been removed. In this way changes of suction pressure were obviated. Portions of shoot, one, two, and three nodes long, were used and the dye solution was allowed to enter through one of the petioles of the uppermost pair of leaves, all other cut surfaces having been sealed with vaseline. The arrangement is shown in Fig. 7. The filter candle was thoroughly impregnated



FIG. 7. The method of attaching the evaporimeter cup to the pieces of stem is shown. The arrow indicates the petiole through which the dye was introduced.

and filled with water before fitting to the shoot, and coloration of the vascular bundles above the supply petiole was taken to indicate the presence of a vascular interconnexion; this was found only in those shoots having three pairs of leaves. The other shoots were examined below the supply petiole and bundles were found to be stained, indicating that the dye had entered the shoot.

These experiments show therefore that the leaves on a shoot bearing up to three pairs of leaves are all isolated from one another, but in a shoot bearing four pairs of leaves the uppermost two pairs are connected together in the region of the lowest node.

An anatomical investigation was now undertaken to verify these findings and locate the position of the interconnexions. The main shoots used in the previous physiological analysis were much too long for microtome work because at least four nodes needed to be examined, and for this reason axillary growths were used which included four nodes in  $2\frac{1}{2}$ –3 in. of stem as compared with 7–10 in. of the main stem. The material was cut into short lengths conveniently marked so that they could be correctly orientated relative to one another, embedded in paraffin wax by the usual methods, and sectioned.

Drawings of suitable sections were made, and each xylem vessel was traced down the length of the stem. From these drawings the vascular system was reconstructed as shown in Fig. 8. The nodes are labelled A–D and the bundles of each node 1–6, the median traces being ringed. It will be seen that the bundles of the leaves of nodes A and B become associated in the region of node D, while the traces of the other leaves remain separated from one another during their course down the stem, thus corroborating the results of the physiological work. The fusion of A<sub>5</sub> and A<sub>6</sub> with the left and right branches of B<sub>4</sub> are illustrated in Fig. 9 (I–VII), which are drawings of the xylem elements of these bundles at the different consecutive levels in the stem indicated by the corresponding Roman numerals. A brief description of the salient features at the various levels follows.

*Fig. 9* (I) The xylem elements of the bundles are widely separated from each other just prior to the entry of the bundles of node C into the stele.

(II) Shows the xylem groups subsequent to the entry of node C bundles. There is an attenuation of B<sub>4</sub> xylem and a close approximation of the groups to each other. The bundles of node C are not shown as the two nearest (C<sub>5</sub> and C<sub>6</sub>) fall outside the bundles A<sub>5</sub> and A<sub>6</sub> as is shown in Fig. 8.

(III) The bundle B<sub>4</sub> is in process of bifurcation.

(IV) The close approximation of the left branch of B<sub>4</sub> to A<sub>5</sub> is seen; the latter has now proliferated to three elements. At this stage the two groups are separated by only one thickness of parenchyma, which is yet sufficient to prevent the passage of water between the two traces.

(V) Shows the composite xylem group A<sub>5</sub>B<sub>4</sub>L. The fusion was, in fact,

effected by the development of a xylem vessel in place of the intervening parenchyma.

- (VI) The entry of bundle D<sub>4</sub> into the stele is seen, and the close approximation of the right-hand branch of B<sub>4</sub> to A<sub>6</sub>.
- (VII) Shows the contact between A<sub>6</sub> and one of the vessels of B<sub>4</sub>R; D<sub>4</sub> is now almost included in the stele.

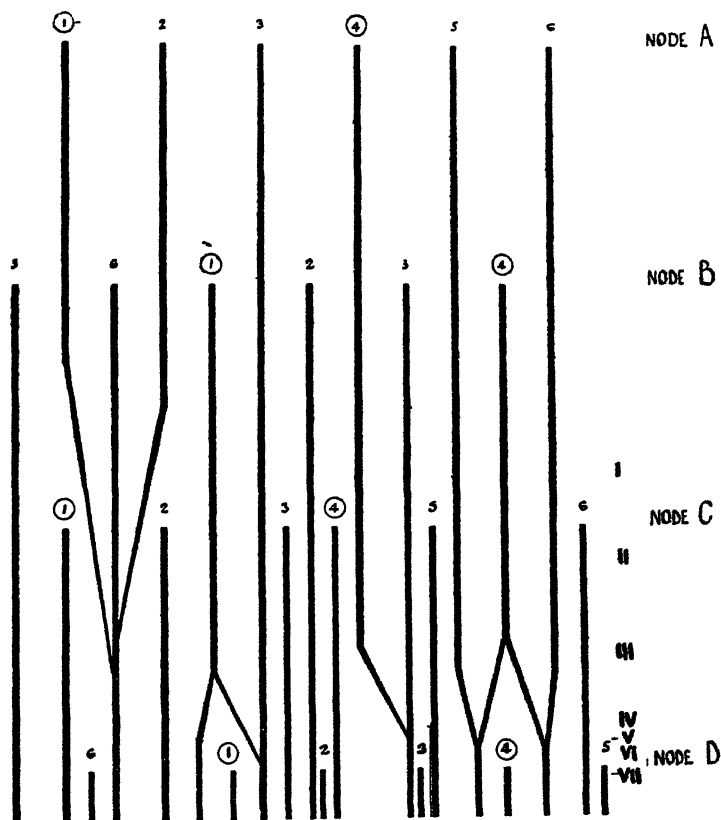


FIG. 8. A reconstruction of the vascular system of the primary shoot from serial sections. The levels at which the various sections (I-VII) were taken are indicated in the diagram.

## DISCUSSION

The possibility of elucidating the primary anatomy of *Eupatorium* resulted from the fortuitous selection of a plant with few vascular interconnexions. The presence of large numbers of fusions would have rendered the interpretation of results very difficult, as shown by similar experiments by Rehm (1935) on *Impatiens Balsamina* and *I. Roylei*, in which he found that water was absorbed with equal facility from both ends of the shoot owing to the frequent fusions between leaf-trace bundles.

Again in searching the literature for the anatomy of the plant used, that of *E. cannabinum* was found recorded by Vuillemin (1884) and is quite different from the present species; it comprises a number of cauline bundles which take a sinuate course in the stem, and from these leaf-trace bundles separate at intervals.

That the present type of vascular structure is not unique, however, is apparent from the description of the anatomy of *Helianthus annuus* by Priestley and Scott (1936) who record that, in the region of  $\frac{3}{8}$  phyllotaxy, 'The three bundles of the trace ran vertically down through eight internodes but at the ninth node below a leaf was inserted which interfered with the continuation of the vertical course, though the incoming leaf was not inserted on the same orthostichy. One of the lateral injected bundles forked above the median of the incoming leaf trace, the injected median forked above an incoming lateral, whilst the second injected lateral continued down undivided.' It would appear therefore that the nine leaves mentioned are isolated from one another, and experiments on *Helianthus* similar to those recorded should be of interest.

In examining the sections it was noted that the vascular strands of the leaves above node A, i.e. those still in the bud, were not differentiated at all but were present merely as procambial strands, and it follows that the development of the vascular elements was very rapid since the strands of the leaves below these (i.e. those of node A) had developed through two internodes and had joined the strands of the leaves of node B. The development of the traces must also have taken place from the base of the petiole upwards and downwards, because the vascular bundle comprised more xylem vessels in this position than it did at the junction of the vascular bundles, and this downward attenuation of the bundles was characteristic of them all.

The water-supply to these very young leaves is therefore a matter of some interest, since where no xylem is developed they must obtain water by withdrawing it from the surrounding parenchyma. The volume required for transpirational purposes is most likely very small since the length of the laminae at this stage in their development is 3–4 mm., and they are closely packed in the bud, thus being maintained in a still and humid atmosphere. The stomata, however, are already developed, but whether or no they are then functional is unknown.

An increase in the size of the lamina, and its related increase in the volume of water required for vacuolation and transpirational purposes, must be connected with the development of the vascular bundles down the stem, and until these meet and fuse with those of the node below the water requirements of the leaves must still be met from the stem parenchyma. The downward extension of the vascular system brings the vessels into contact with larger numbers of parenchyma cells from which water may be withdrawn, and by the time that the volumes of water are so large that they cannot readily be supplied by the parenchyma, the vessels have meanwhile differentiated down to fuse with the traces of node B which continue down to that part of the stem where secondary thickening is proceeding. Even at this stage in the ontogeny

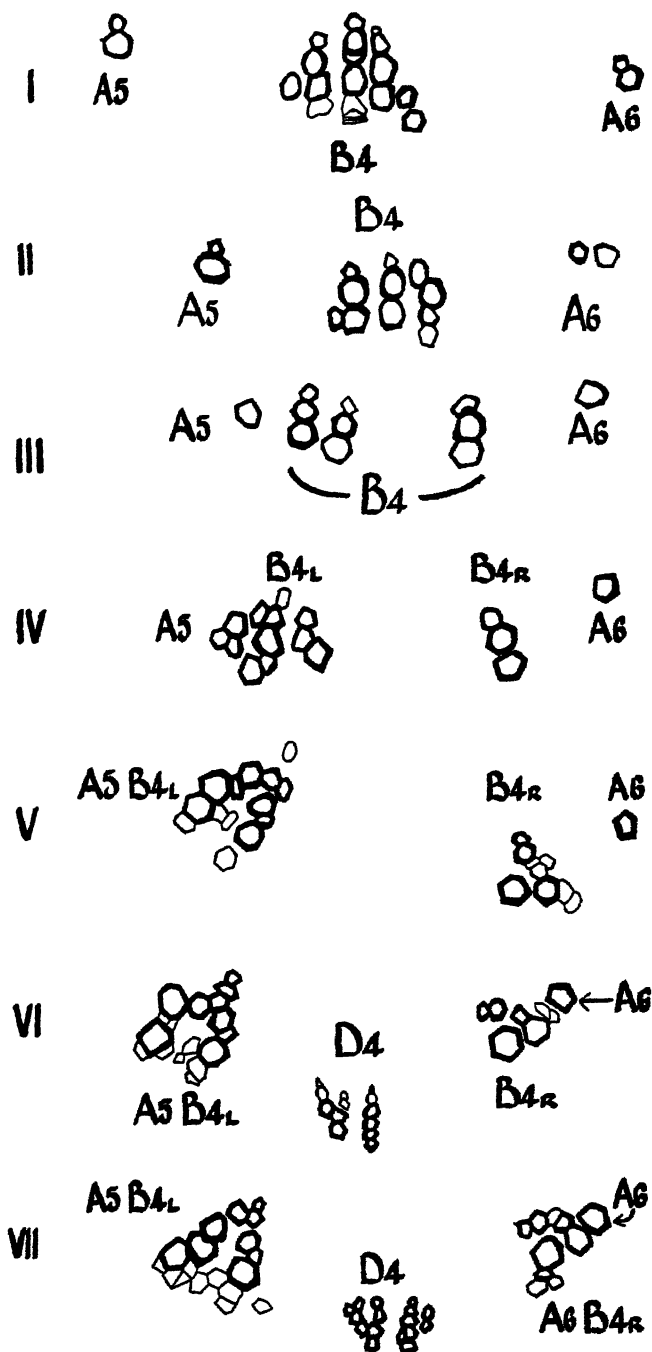


FIG. 9. Camera-lucida drawings of certain of the bundles of the primary shoots to show their course down the stem and their anastomoses. For details see text.

of the leaves the quantity of water required must be quite small, for it was noted that the three bundles of the node A leaves were composed of only two small vessels each.

The factors determining this particular type of development are at present unknown. Evidence from experimentation on the regeneration of severed vascular bundles (Freundlich, 1908; Simon, 1908) and the development of buds on a callused surface (Simon, 1908, 1930) seems to indicate that the tracheids which develop to bridge the gap do so apparently haphazardly and join the nearest vessels in contact with the water-supply. This is also the case with the axillary growths of *Eupatorium*; at the base of a lateral shoot the vascular strands fuse to form a number of larger strands and these are connected to the vascular structure of the main stem by way of a mass of tracheids.

The presence of independent strands traversing a definite number of internodes seems to indicate that the conditions in the apex of the shoot differ radically from the fortuitous vascular anastomoses occurring during regeneration of the vascular system after wounding, for in the former case the course of differentiation is not determined by a steep gradient of water shortage as it appears to be in the latter. On the contrary, in the apex the whole course of development bears reference to a future state of affairs when the leaves shall have become functional in transpiration. That the fusion of the bundles is due solely to exigencies of space cannot be maintained, for in these circumstances fusions might reasonably be expected to occur between any of the eighteen bundles present in this region of the stem and not specifically between those of nodes A and B.

It would appear therefore that the leaves situated on the primary shoot are isolated from one another, becoming interconnected in that part of the stem where secondary thickening is taking place. The primary structures may be regarded as being seated on the secondary development, and the fusion takes place when the increasing volumes of water required by the leaves for transpirational purposes can be supplied through the secondary xylem of the older parts of the stem.

The information here discussed has a bearing on the work of Roach (1939), who has developed a practical field method of diagnosing and treating mineral deficiency diseases in a wide range of plants by injecting leaves and shoots either through a severed petiole or through the cut end of a shoot. The most interesting distribution of dyes to the remaining leaves on the shoot was obtained when entry through a petiole was employed, the area of the leaf coloured by the dye being related to its position on the stem relative to the supply petiole. How these distributions arise is not known in detail as the anatomy of the plants was not closely examined. The elucidation is complicated because secondary thickening begins early in the plants employed (Apple, Pear, Raspberry) and the shoots remained on the plant, so that the normal water-supply from the roots to the leaves continued during the progress of the injection.

Since the leaf traces of the supply petiole were presumably linked through

the secondary xylem with all the other leaf traces it would be expected that a general permeation of the whole shoot would result, which is contrary to the experimental findings of Roach. Further work needs to be done to rationalize this method of diagnosis by injection, and particularly to ascertain the behaviour of shoots severed from the plant in which the supply of water is canalized through the supply petiole.

Even after fusion of the leaf traces with the secondary xylem it would appear that there are 'preferential' channels of water movement and these may represent the original fusions of the leaf traces of the kind investigated in this present work.

#### SUMMARY

Experiments were undertaken to investigate the resistance to the movement of water across the medullary rays of the primary shoot of *Eupatorium adenophorum*; these showed that the resistance was greater than could be overcome by the maximum suction pressure of the leaves.

When water was supplied only through a single delaminated petiole on portions of the primary shoot bearing two and three pairs of leaves respectively, all the leaves wilted.

When water was supplied only through the cut apex of detached portions of shoots bearing one and two pairs of leaves these similarly wilted.

When water was supplied through the apex of detached shoots bearing three pairs of leaves the uppermost pair remained turgid while the lower two pairs wilted. A vascular interconnexion thus existed between the leaf traces of the uppermost pair of leaves and the bundles in that part of the stem above their insertion. The position of the interconnexion was deduced to be in the region of the lowermost node.

An anatomical investigation confirmed these deductions.

The author wishes to acknowledge his indebtedness to Professor F. G. Gregory, F.R.S., for the suggestion of the problem and for his sustained interest throughout the investigation.

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# Studies in Vernalisation

## XI. The Effect of Date of Sowing and of Excising the Embryo upon the Responses of Petkus Winter Rye to Different Periods of Vernalisation Treatment

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With four Figures in the Text

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### I. INTRODUCTION

EXCISED embryos of winter rye can be successfully vernalised while germinating on an agar medium containing sugar, but in the isolated embryo a 'lag' period occurs at the beginning of the process; once begun, however, progress takes place at the same rate as in the whole grain (Purvis, 1944). It was inferred in this earlier paper that excised embryos need to synthesize, from the simple materials provided in the medium, some prerequisite of vernalisation, which in the intact grain is derived from the endosperm or aleurone layer. Since, however, the data there presented were obtained in different years, no final conclusion was warranted; and in this paper further

experiments are described which confirm the existence of this 'lag' period. In the course of these experiments it became evident that the date at which the vernalised material is planted exerts a profound effect upon the responses of both intact grain and excised embryos to vernalisation treatments of various durations. An attempt is made in this paper to analyse the factors concerned in this effect of sowing date.

## II. THE EFFECT OF VERNALISATION FOR DIFFERENT PERIODS OF TIME ON INTACT GRAIN AND ON EXCISED EMBRYOS

### (a) *Methods*

A technique for vernalising excised embryos has already been described (Purvis, 1944), and substantially the same method was used in the experiments recorded here. In 1942, however, it was found that sterilization by alcohol *after* soaking the grain gave inadequate protection against fungal invasion; since at that time it was thought inadvisable to prolong the soaking period by sterilizing with calcium hypochlorite solution, the dry grain was thoroughly shaken with absolute alcohol for 2 minutes and quickly dried in a stream of filtered air. This change in method did not affect the vernalisation response, but the germination of a few individual embryos was completely inhibited; probably this is related to slight sprouting. From each lot of sterilized grain parallel series of whole grains were sown in moist sand. These were vernalised in the same conditions of temperature as the various series of excised embryos reared in test-tubes. In 1941 seedlings treated in these ways were planted out in sand-culture pots (Purvis, 1934) on May 20, and the plants which had not already flowered were dissected on October 7, 140 days after planting. In 1942 the growing season was extended to 216 days by planting during the first week in April and delaying the final examination. To avoid the possible vernalising effect of low temperature outdoors, the seedlings were kept in a cool greenhouse until the first week in May. Even with the long growing-period employed some plants failed to reach the stage of anthesis. The flowering behaviour of the various series is therefore expressed as the mean of the 'scores' of individual plants, including thus data for both flowering and non-flowering plants (Purvis, 1947).

### (b) *Results*

The 'scores' in the two experiments are given in Table I and in Fig. 1; they were, of course, much higher in 1942 owing to the extended growing-period. In both years, however, the progress of vernalisation in excised embryos clearly lags behind that in whole grain by 11–14 days, whereas, after the termination of the 'lag' period, the rate is the same in both.

The form of the curve relating duration of vernalisation treatment to flowering response is modified not only by excision but also by the different conditions obtaining in the two years. In 1942 the intact grain responded to the shortest low-temperature treatment (4 days), whereas in 1941 whole grain treated for 14 days showed no effect of vernalisation and this ineffective period

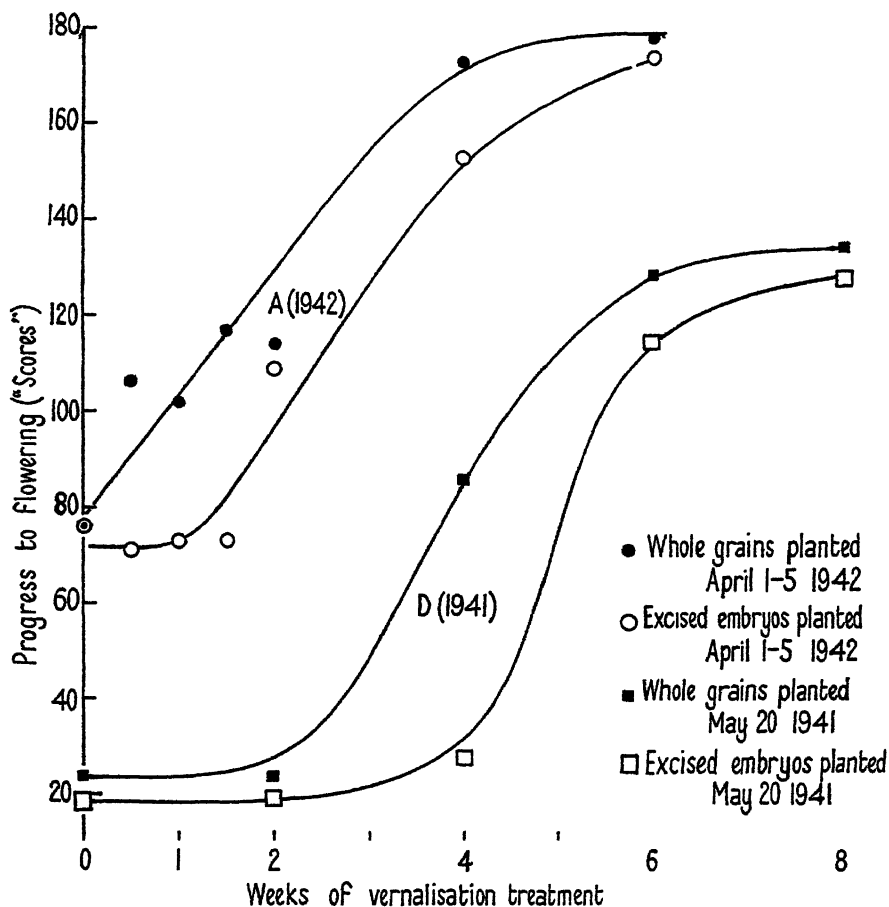


FIG. 1. Flowering behaviour ('scores') of whole grain and of excised embryos in relation to duration of vernalisation treatment. Plants grown in pots. *A*, 'scores' determined 216 days after planting on April 1-5, 1942. Solid circles, whole grain: hollow circles, excised embryos. *B*, 'scores' determined 140 days after planting on May 20, 1941. Solid squares, whole grain: hollow squares, excised embryos.

TABLE I

*Effect of Different Durations of Vernalisation on Whole Grain and on Excised Embryos of Rye (Number of Replicates in Brackets)*

Duration of treatment in weeks.	Planted May 20, 1941. 'Scores' at 140 days.		Planted April 1-5, 1942. 'Scores' at 217 days.	
	Whole.	Excised.	Whole.	Excised.
0	24±1.1 (19)	19±1.6 (13)	76±15.9 (10)	76±9.2 (11)
$\frac{1}{2}$	—	—	106±9.1 (12)	71±10.2 (11)
1	—	—	102±7.5 (9)	73±11.3 (12)
1½	—	—	117±8.2 (13)	73±13.0 (8)
2	24±1.3 (20)	19±0.9 (19)	114±7.3 (14)	109±10.3 (15)
4	86±6.6 (17)	28±5.2 (18)	173±1.3 (10)	153±7.2 (10)
6	129±0.8 (17)	115±3.8 (14)	178±1.5 (10)	174±2.2 (15)
8	135±0.6 (19)	129±1.4 (18)	—	—

extended to nearly 4 weeks when the embryos were excised. In the experiment of 1941 there was thus a 'lag' period in the whole grain as well as in the excised series. Further, in the intact grain, in 1942, the vernalisation effect approached a maximum after 6 weeks' treatment, whereas in 1941 treatment for 8 weeks was not yet maximal. As the vernalisation technique was unchanged in the two years, the difference must be attributed to environment after planting.

*(c) Effect of sowing date on responses of excised embryos (1943)*

It has been stated that despite the disparity between the two curves in Fig. 1 for whole grain, the excised embryos lagged behind in both years. To confirm this, in 1943 excised embryos were planted out on three occasions following vernalisation treatment of different durations on a medium containing 2 per cent. sucrose. The result of a parallel experiment using a medium without sugar has been described elsewhere (Purvis, 1947). At the same time the effect of sowing date on the vernalisation response of whole grain was investigated in a plot experiment, which is described in a later section of this paper. In this experiment whole grain was planted on four occasions, namely, April 5, April 19, May 3 and May 17, following vernalisation treatment for different periods. Seedlings from each of these series were planted in sand-culture pots as in earlier work, for comparison with the excised embryos which were too small and fragile for planting direct in soil. It was not found possible to plant the excised embryo series on the same days as the whole grain: the planting dates were, accordingly, April 7, May 4, and May 24.

The first two series are thus comparable with the first and third plantings of whole grain, while the third series was planted 1 week later than the corresponding whole-grain series. Replication was adequate except in one series in which fungal contamination reduced survival. Both whole-grain and excised embryos planted on April 5 were kept in a cool greenhouse until May 1; later plantings were directly in the open. In several series of excised embryos the seedlings were too small to be planted in pots immediately vernalisation treatment ended, and were therefore exposed to light at room temperature while remaining on the agar medium until they were large enough to handle. This period, generally 3 to 4 days, is included in the time required to flower.

The mean 'scores' of the various series are given in Table II, and in Fig. 2 the parallel series of whole grain grown in sand cultures (see Table VIII, p. 194) are included for comparison. It should be remembered here that the two curves marked 'D' are not quite comparable, since the whole grain (solid squares) was planted a week before the excised embryos (open squares).

Clearly the flowering behaviour of plants grown from excised embryos is influenced by the date of planting out in much the same way as that of whole grain plants, and this accounts for the difference between the results in two consecutive years shown in Fig. 1. For each planting date with the same external conditions a comparison of excised and whole grain shows in each case, moreover, a delay in the inception of the vernalisation process in the

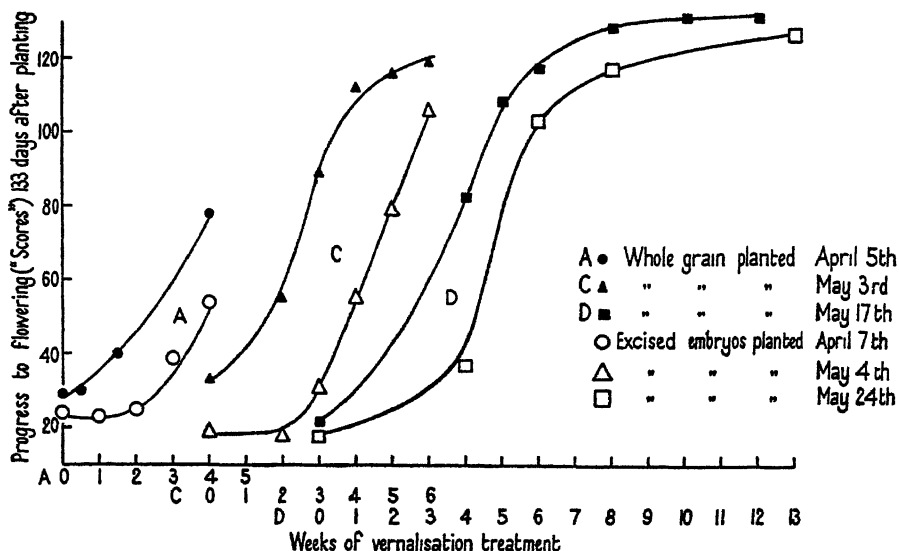


FIG. 2. Effect of planting date on flowering response to vernalisation ('scores' determined 133 days after planting) of whole grain and of excised embryos given treatments of different durations. Plants grown in pots. *A*, whole grain planted April 5 (solid circles), excised embryos planted April 7 (hollow circles); *C*, whole grain planted May 3 (solid triangles), excised embryos planted May 4 (hollow triangles); *D*, whole grain planted May 17 (solid squares), excised embryos planted May 24 (hollow squares). The scales along the abscissa are staggered to show the approximate intervals between the planting dates.

TABLE II

*Petkus Winter Rye. Effect of Planting Date and Duration of Vernalisation on Flowering Behaviour of Plants grown from Excised Embryos (Replication in Brackets)*

Date of planting: Duration of vernalisation in weeks.	April 7.	May 4.	May 24.
	'Scores'.		
Unvernalised	$24 \pm 1.7$ (24)	$19 \pm 1.1$ (22)	$18 \pm 1.0$ (30)
1	$23 \pm 1.1$ (31)	—	—
2	$25 \pm 2.2$ (27)	$18 \pm 2.5$ (4)	—
3	$39 \pm 4.4$ (25)	$36 \pm 6.2$ (20)	—
4	$54 \pm 5.3$ (25)	$55 \pm 6.7$ (22)	$37 \pm 8.0$ (11)
5	—	$79 \pm 7.9$ (15)	—
6	—	$106 \pm 1.4$ (15)	$104 \pm 6.1$ (15)
8	—	—	$118 \pm 0.6$ (24)
13	—	—	$128 \pm 0.6$ (24)

excised embryos relative to whole grains, followed later by progress at the same rate in both (Fig. 2). The results given in Fig. 1 are thus entirely confirmed.

(d) *The significance of the 'lag' period*

It has been suggested that the 'lag' period represents the time required for the embryo to synthesize substances requisite for the vernalisation process.

No systematic attempt has been made to supply specific substances in the medium and in this way to eliminate the 'lag' period, but it has been found that neither dried yeast, hetero-auxin, nor ground rye meal has such an effect. In this connexion, however, it is necessary to consider the complexity of the processes whereby the embryo absorbs endosperm material. Sucrose which is used in the medium in these experiments is the normal end-point (Brown and Escombe, 1897) of a complex process of dissolution. The embryo is unable to assimilate starch either when supplied in the medium or from sterile potato tissue (unpublished observation). Further, even when contact with its own endosperm is unbroken the embryo fails to assimilate normally if the aleurone layer is removed or even separated from the embryo by ringing (Schander, 1934). Grains thus treated are vernalised by low temperature at about the same rate as excised embryos kept without sugar. Thus it might be possible to eliminate the 'lag' period by adding to the medium degradation products of endosperm. Other attempts to shorten this period were made by keeping the embryos in contact with sucrose at room temperature for several days prior to vernalisation treatment; it seemed reasonable to suppose that this part of the process might be independent of low temperature and might indeed be accelerated at higher temperatures (Table III). In no case, however, was the 'score' significantly increased by the preliminary treatment with sugar. Thus either low temperature is a prerequisite of the synthesis, or at high temperature the increased demand for growth of the embryo is a competing factor.

TABLE III

*Petkus Winter Rye. 'Scores' at 133 Days of Plants grown from Excised Embryos vernalised 4 Weeks. Planted May 2-5, 1944 (Number of Replicates in Brackets)*

Days of pre-treatment	0	1	2	3	4
On sucrose at 20° C.	80±4.5 (28)	82±4.1 (29)	80±4.9 (30)	76±4.6 (31)	76±5.9 (22)
Imbibed intact at 20° C. before excision	80±4.5 (28)	94±1.3 (40)	95±2.2 (37)	87±3.6 (34)	88±3.2 (37)
Intact grain vernalised 4 weeks	106±1.4 (18)				

When the excision was delayed until the embryo had been in contact with its own endosperm either for 1 or for 2 days at 20° C. there was a significant advance of flowering; more than half the difference between the response of excised embryos and that of the intact grain was eliminated in this way. Prolonged contact at 20° C. diminished the acceleration until it failed to be significant. This again may be due to competition by growth processes.

When, however, during the preliminary period in contact with the endosperm *low* temperature was maintained, the disparity between excised embryos and whole grain was almost eliminated. In an experiment in 1947 the 'score' (determined 133 days after planting) of whole grain vernalised 5 weeks was 114±1.0 and that of excised embryos also vernalised for 5 weeks on sugar

was  $94 \pm 4.8$ ; when the embryos were excised at the end of 1 week of low-temperature treatment and remained at low temperature on sugar medium for a further 4 weeks, the 'score' 133 days after planting was  $110 \pm 1.6$ . One week of normal contact between embryo and endosperm at  $1.8^\circ \text{C}$ . thus raised the 'score' by 16 units to a level little below that for whole grain. There is thus strong support for the hypothesis to account for the 'lag' period put forward in an earlier paper (Purvis, 1944). *Transfer* of a specific substance to the embryo from the endosperm occurs at both high and low temperature, but at the higher temperature in course of time other factors interfere. The supposed *synthesis* of the same substance by the excised embryo from the simple material in the medium apparently can only occur at low temperature.

### III. PLANTING DATE EXPERIMENT IN 1943 USING INTACT GRAIN

#### (a) *Plan of the experiment*

The aim of this experiment was to determine the effect of the date of planting out on the responses of whole grain to vernalisation treatment of varying durations. By growing the plants in rows in the field instead of in pots increased replication was secured, and thus the high standard errors encountered in previous work with partially vernalised series were substantially reduced (Table IV). Some plants from each series were grown in sand culture for comparison, and also to serve as controls for the 'excised embryo' series which have already been discussed. Planting was at fortnightly intervals, on four occasions, namely, April 5, April 19, May 3 and May 17. In view of the results shown in Fig. 1, with early sowings the shorter durations of vernalisation were investigated in particular, while with later sowing these were not expected to have much effect and so were replaced by longer durations. On each planting occasion the following material was employed: (1) winter rye unvernalsed, (2) winter rye vernalised for six different periods, and (3) unvernalsed spring rye. The full range of treatments may be seen in Table IV.

#### (b) *Vernalisation technique*

Vernalisation treatment was given to unsterilized seed germinating in sand with unrestricted moisture. The grain was sown at fixed depth in dry sand held in uncovered wooden boxes. The sand was moistened by immersion in tap-water, and after 24 hours at room temperature the boxes were transferred to a cold chamber at  $1.1^\circ \text{C}$ . Unvernalsed control series of winter and spring rye were sown in the same way and maintained at room temperature for 2 days before each planting date.

#### (c) *Planting*

A plot 15 ft. wide was divided into two 7 ft. blocks by a path. Each variant was planted in two rows, one in each block, spaced at  $1\frac{1}{2}$  in. between plants (i.e. 56 seedlings per row). The rows were 1 ft. apart; they were not randomized, but the order of sowing was reversed in the two blocks. Guard rows of spring rye were sown at the beginning and end of each planting. There

was some loss due to birds, but this was not heavy and ample replicates remained.

(d) Results

(i) *Progress towards flowering.* The flowering behaviour of the various series is expressed as the mean 'score' attained by each two-row unit 19 weeks after planting (Purvis, 1944). The data are presented in Table IV. Means for the two separate rows are not given: in most series they agree well, and even the largest discrepancy between replicate rows was not statistically significant. The rows of figures in this table demonstrate the effect of planting-out date on the response for various constant durations of treatment; the columns give the effect of duration at various dates of planting. These data are presented graphically in Fig. 3 (curves A–D); for clarity of presentation the scales for the different planting dates along the abscissa are 'staggered' at fortnightly intervals. This also permits the presentation (dotted lines) of the 'sowing-date' effect by joining the 'score' points corresponding with equal periods of vernalisation in the series planted out on successive occasions. A similar curve for unvernalsed spring rye is also presented (S).

TABLE IV

*Petkus Winter Rye, Whole Grain. Effect of Date of Planting on Response to Different Durations of Vernalisation Treatment (Number of Replicates in Brackets)*

Date of planting: Duration of vernalisation in weeks.	April 5.	April 19.	May 3.	May 17.
	'Score' 133 days after planting.			
0	37±2.2 (33)	34±1.4 (80)	29±1.4 (53)	24±0.7 (62)
$\frac{1}{2}$	41±1.7 (56)	—	—	—
1	53±2.2 (64)	39±2.1 (75)	—	—
1½	60±2.9 (48)	—	—	—
2	63±3.4 (53)	45±2.2 (83)	31±1.9 (62)	—
3	—	48±2.7 (70)	44±3.5 (62)	—
4	98±1.1 (62)	89±2.5 (80)	97±3.5 (64)	28±2.1 (63)
5	—	103±1.0 (73)	107±2.3 (64)	61±4.5 (69)
6	107±0.2 (81)	111±0.4 (77)	115±0.6 (55)	108±2.9 (81)
8	—	—	121±0.4 (76)	124±0.4 (87)
10	—	—	—	128±0.4 (81)
12	—	—	—	130±0.3 (92)
Spring rye un- vernalsed	110±0.3 (62)	118±0.2 (76)	122±0.4 (62)	127±0.4 (68)

The following results are apparent:

1. In unvernalsed winter rye delay in sowing reduces the 'score' and delays progress towards flowering. This is, no doubt, associated with 'natural vernalisation' following earlier times of planting out.
2. In unvernalsed spring rye the opposite effect of sowing date is seen; late sowing accelerates the rate of progress towards flowering.

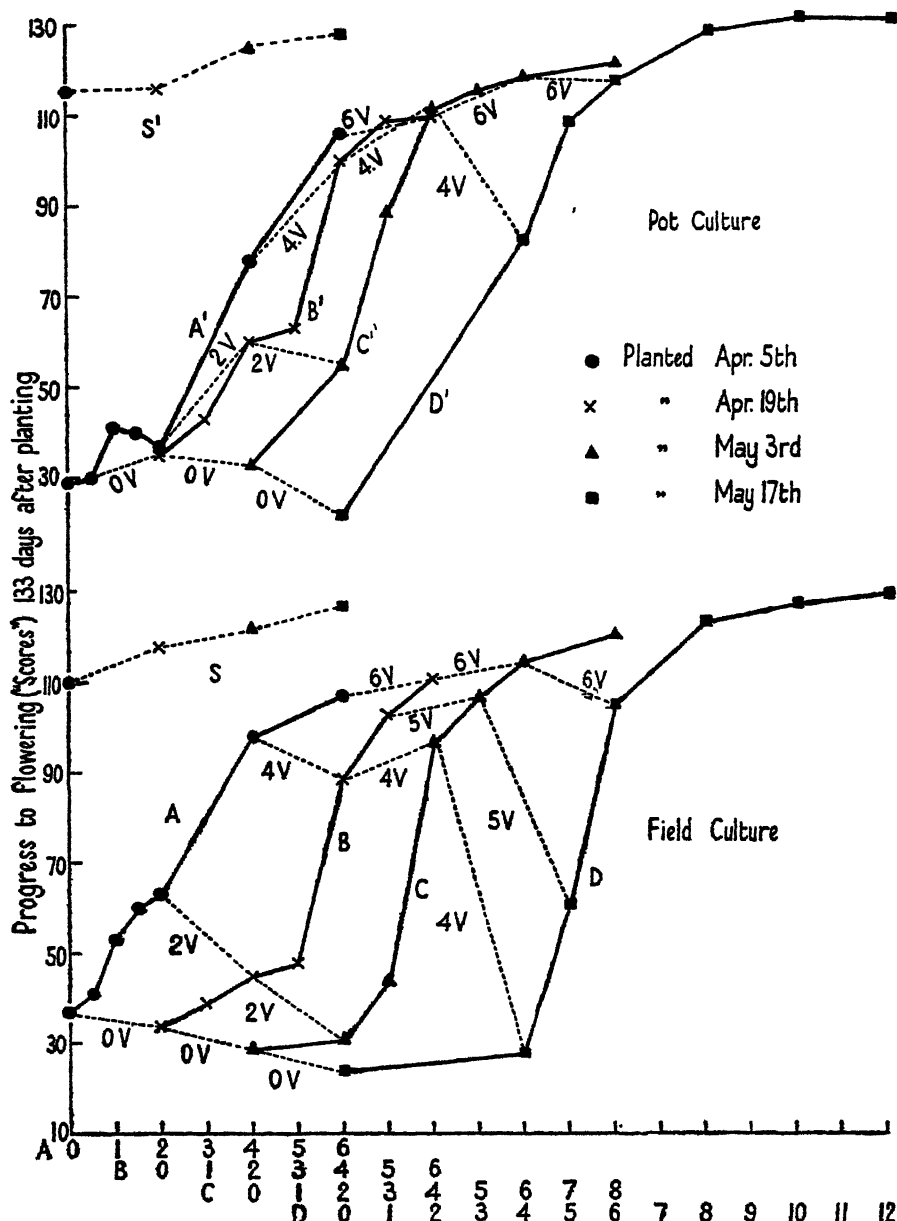


FIG. 3. Effect of planting date on flowering response to vernalisation ('scores' determined 133 days after planting) of whole grain given vernalisation treatments of different durations. *A*, winter rye, planted in field and *A'* in pots, on April 5; *B*, winter rye, planted in field and *B'* in pots, on April 19; *C*, winter rye, planted in field and *C'* in pots, on May 3; *D*, winter rye, planted in field and *D'* in pots, on May 17. The scales along the abscissa are staggered to show the intervals between the planting dates: the broken lines indicate the effect of the date of planting on the flowering of equally vernalised plants. The broken line at *S* shows the effect of the date of planting on Petkus spring rye.

3. The series of curves ('duration curves') relating the 'scores' attained to the duration of low-temperature treatment displays a progressive change in form as the sowing date is advanced. In the earliest sowing (April 5) the curve is linear from the start, whereas the subsequent curves become more and more sigmoid. In the sowing on May 3 no effect on the 'score' is seen after 2 weeks of vernalisation, and this 'lag' in the effect of vernalisation is increased in the sowing on May 17 to 4 weeks. The salient features of the two contrasting curves shown in Fig. 1 for experiments of 1941 and 1942 are thus reproduced in this experiment of 1943, and the variation in form of the curves is seen to be due to sowing date.

4. The dotted lines in the diagram show that after 2 weeks of vernalisation the delay in sowing date has an even greater effect than in unvernalsed winter rye. After 4 weeks of vernalisation delay in sowing has no consistent effect on the 'score' when the sowing date is delayed from April 5 to May 3, but a further delay in sowing to May 17 leads to a great reduction in progress to flowering. After 6 weeks of vernalisation the delay in sowing progressively accelerated flowering until the last sowing which resulted in a slight retardation. After 8 weeks' vernalisation, however, delay from May 3 to May 17 in planting resulted in accelerated flowering (as in spring rye).

5. A further effect of sowing date is seen in the maximum score attained. Seedlings planted out on April 5 attain a maximum 'score' after 6 weeks of vernalisation, and the broken line for 6 weeks' duration shows that the 'score' is increased only slightly by delaying sowing; but in the later sowings the maximum 'score' has not been attained by 6 weeks' vernalisation and longer treatment at low temperature still further increases the 'score', so that the value after 12 weeks of vernalisation in the sowing of May 17 equals that of spring rye. The upper limit of the intermediate sowings is not known as vernalisation was not in these cases carried on for 12 weeks.

(ii) *Effects on vegetative growth and on ear production.* The number of tillers per plant at the end of the experiment, shown in Table V, is restricted by the close spacing, as shown by the freer tillering in rows with poor replication.

TABLE V  
*Effect of Date of Planting on the Number of Tillers per Plant at End of Experiment (Number of Replicates in Brackets)*

Date of planting: Duration of vernalisation in weeks.	April 5.	April 19.	May 3.	May 17.
0	18.3 (35)	7.1 (86)	8.5 (84)	8.9 (64)
1	8.1 (64)	6.0 (76)	—	—
2	8.7 (53)	6.5 (86)	8.0 (70)	—
3	—	6.3 (74)	5.4 (62)	—
4	4.4 (57)	3.1 (78)	3.7 (69)	7.2 (65)
5	—	2.7 (78)	3.5 (68)	4.8 (73)
6	4.7 (85)	3.8 (78)	3.3 (58)	3.7 (64)
8	—	—	3.7 (75)	3.4 (87)
Spring rye	4.5 (77)	3.6 (80)	4.3 (60)	4.0 (72)

Within the limits thus imposed, however, the reduction in tillering which accompanies early flowering is shown for all sowing dates; the extent of tillering is clearly conditioned by the factors governing flower induction.

The final condition of these tillers is also determined by the flowering response of the plant. Where the response to vernalisation is large, all the few surviving tillers bear ears. In plants which have not responded to vernalisation all the numerous tillers have continued to produce leaves and remain green, while extension of the stem fails. Between these extremes, where vernalisation is partial, the plants exhibit a number of green tillers, and produce also a small number of ears. This additional expression of 'lateness' is not recorded in the 'scores' in Table IV, where only the earliest ear of each plant is considered. It is, however, shown in Table VI, where the number of ears per *eared plant* (i.e. excluding plants which failed to produce emergent ears) is seen to increase as flowering is accelerated. Thus a diminished response not only retards flowering but also decreases the total flower production: this results in a graded relation between yield and duration of vernalisation; consequently it is important in practice to carry the process of vernalisation to completion.

TABLE VI

*Petkus Winter Rye. Number of Emergent Ears per Plant (excluding completely Vegetative Plants) in relation to Duration of Vernalisation (Replicate Numbers in Brackets)*

Sowing date: Duration of vernalisation in weeks.	April 5.	April 19.	May 3.	May 17.
0	1.2 (5)	1.2 (23)	—	—
1	1.5 (44)	1.3 (33)	—	—
2	1.8 (36)	1.2 (42)	1.5 (6)	—
3	—	1.7 (33)	1.6 (19)	—
4	3.3 (57)	2.1 (73)	2.9 (62)	1.0 (4)
5	—	2.5 (78)	2.8 (67)	2.0 (36)
6	4.3 (85)	3.7 (78)	3.2 (58)	2.6 (55)
8	—	—	3.5 (75)	3.3 (87)

When vernalisation is complete every tiller which survives the onset of flowering in the main axis comes into ear, including even late tillers which arise after earlier ears are fully ripe; and furthermore, repeated removal of ears as they begin to shoot fails to reduce the flowering potential which has been established during vernalisation treatment. This was demonstrated in 1938 when all shooting ears were excised from completely vernalised plants of winter rye on two occasions, the first 4 weeks, the second 6 weeks after planting (Table VII). The mean number of days from planting to the first anthesis of each plant is given in each case. In the mutilated plants the small tillers present at the second excision proceeded to shoot and flowered about 40 days later.

TABLE VII

*Days from Planting to Anthesis of Vernalised Plants (sown May 2) from which all Ears had been removed after 4 and again after 6 Weeks (Number of Replicates in Brackets)*

Vernalisation period	12 weeks.		8 weeks.	
	Ears removed.	Untreated.	Ears removed.	Untreated.
Days to anthesis	82±0.3 (3)	61±0.4 (19)	80±0.4 (5)	64±0.3 (20)

It appears then that *every* growing point in the fully vernalised plants is in a vernalised condition, whereas in partially vernalised plants the factor which determines flowering is quantitatively limited and is exhausted after the earing of one or two tillers.

(e) *Parallel experiment with plants grown in pots*

As stated earlier, seedlings from each series were also grown in pots for comparison with excised embryos grown in the same manner. This afforded the additional advantage that the earliest series (planted on April 5) could be protected at first from 'natural' vernalisation by keeping the plants in a cool greenhouse. The 'scores' for these plants in pots determined 133 days after planting are given in Table VIII and Fig. 3 (A'-D').

TABLE VIII

*'Scores' of Plants vernalised for Different Periods, and planted out in Pots at Fortnightly Intervals (Replicate Numbers in Brackets)*

Sowing date: Duration of vernalisation in weeks.	April 5.	April 19.	May 3.	May 17.
	'Scores' 133 days after planting.			
0	29±2.3 (11)	35±3.7 (12)	33±5.1 (13)	22±2.6 (11)
$\frac{1}{2}$	30±2.1 (10)	—	—	—
1	41±5.5 (8)	43±6.0 (12)	—	—
1 $\frac{1}{2}$	40±5.2 (9)	—	—	—
2	37±5.1 (11)	60±5.8 (13)	55±7.6 (12)	—
3	—	63±6.8 (14)	89±2.7 (6)	—
4	78±5.3 (15)	100±1.3 (14)	112±1.8 (13)	83±6.4 (13)
5	—	109±0.5 (15)	116±1.1 (15)	109±2.6 (13)
6	106±0.7 (15)	110±1.1 (13)	119±0.8 (14)	118±1.4 (15)
8	—	—	125±0.5 (15)	129±0.5 (15)
10	—	—	—	132±0.5 (15)
12	—	—	—	132±0.6 (14)
Spring rye	115±0.5 (15)	116±0.4 (15)	125±0.4 (15)	128±0.6 (15)

With the earliest sowing when the plants in pots were protected from natural vernalisation, progress to flowering was substantially slower than in the parallel series grown in the field (cf. Table IV). With later sowings, however, culture in pots has accelerated flowering throughout, and this is especially marked in the last sowing after incomplete vernalisation. The acceleration in pots has been observed in three further experiments which will be described

in a later paper; the accelerations due to pot as compared with field culture in four different years are given in Table IX.

TABLE IX  
*Acceleration in 'Scoring Units' due to Culture in Pots*

Year.	Planting date.	Duration of vernalisation in weeks.						Spring rye.
		0	2	3	4	5	6	
1943	April 5	-8	-26	—	-20	—	-1	+5
(from tables	April 19	1	15	15	11	7	-1	-2
IV & VIII	May 3	4	24	35	15	9	4	3
above)	May 17	-2	—	—	55	48	10	1
1944	May 3	7	—	—	41	18	-2	—
	May 17	-1	—	42	49	—	12	—
1946	May 1	7	16	25	22	16	6	5
1947	May 1	1	20	11	31	—	5	—

Beyond doubt culture in pots results in more rapid progress to flowering in all sets except in those which were protected from natural vernalisation, and it is clearly necessary to take this fact into account when analysing the factors concerned in the effect of sowing date. It is noteworthy that the difference is greatest when vernalisation is incomplete.

(f) *Effects of environmental factors after planting on the progress towards flowering*

The vernalisation technique is sufficiently well controlled to ensure that grain vernalised for a particular period on different occasions is vernalised to the same extent. There must therefore be a unique relation between the duration of treatment and the degree of vernalisation. Were a direct method available of assigning a quantitative measure to the degree of vernalisation in the grain, the curve relating the variables could be determined directly, but the relative effects of different duration treatments can at present only be assessed from the flowering behaviour of the plants ('duration curves'). There is, however, an interaction of progress towards flowering and the environmental factors, and the eight curves shown in Fig. 3 may be regarded as modifications of the true 'duration curve' whose form is not known. There is considerable evidence apart from the curves in the figures that the process of vernalisation is autocatalytic (Purvis, 1944, 1947) and that the 'fundamental' curve is sigmoid in form. It is therefore of some interest in studying the process to determine which of the eight curves is most likely to approximate to the primary curve, and to examine how the modifying factors operate. Progressive delay in sowing involves steadily lengthening days and a somewhat irregular rise in temperature. Comparing plants in the field and in pots, these were grown under the same day-length, and apart from possible nutritional differences which have been shown to have little effect on the flowering of rye (Purvis, 1934), the observed differences in behaviour must have been due to effects of temperature. Up to the time of shooting, the stem apex is

underground, and is thus subject to greater extremes of temperature when grown in pots. The interaction of sowing date and vernalisation has been studied by von Denffer (1939) using white mustard, whose vernalisation response is evident only in conditions of short days, and also barley (Eckendorffer Mammuth) in which, as in other temperate cereals, the effect of vernalisation is masked by short days. This variety of barley is intermediate in character between a winter and a spring type, for it will ear after early spring sowing, but nevertheless responds in a marked degree to vernalisation treatment.

In his preliminary studies von Denffer provided different day-lengths by sowing at intervals in the spring. In this barley, as in winter rye, response to short periods of vernalisation was greater after early than late sowing while with long periods of vernalisation the reverse is true. The behaviour of this variety of barley differs from that of winter rye described in this paper in two respects: (1) in the barley short periods of vernalisation are much more effective; (2) there was no acceleration after a period of induction by short days. These differences are no doubt due to the 'intermediate' type of barley variety used. Von Denffer found that even in artificially controlled days of constant length there remained a sowing date effect in both mustard and barley which must be attributed to associated temperature differences. Lojkin (1936) working with two varieties of winter wheat (Turkey Red and Leap's Prolific) records an effect of sowing date similar to that obtained in rye. The minimal effective period of vernalisation treatment was shortest with early sowing, while the response to complete vernalisation was augmented by late sowing.

(i) *The effects of length of day.* It is an established fact (Purvis, 1934; Purvis and Gregory, 1937) that in unvernalsed winter rye flower initiation is accelerated by short days, although long days are required to complete the development of flowers. Completely vernalised winter rye, however, resembles in all respects a long day plant, and flower initiation is already determined by low-temperature treatment. Further, it is only during the early stages of development that short days can accelerate flowering; when all the labile primordia have been differentiated short days uniformly retard earing (cf., however, Sande Bakhuyzen, 1947, p. 203). The period after planting during which the plant is responsive to acceleration by short days decreases therefore as the duration of the previous vernalisation increases. It has, moreover, been shown (Purvis and Gregory, 1937) that the effect of days of 10 hours' duration reaches a maximum value after 6 weeks' exposure, and that the minimal effective exposure is 4 weeks. The critical day-length, if any, for flower initiation in winter rye is not known; it is, however, with certainty, more than 10 hours.

Von Denffer (1939) found that by increasing the day-length from 16 to 24 hours flowering in Eckendorffer Mammuth barley was accelerated. McKinney and Sando (1933, Table II, p. 171) obtained earlier flowering in a large range of wheat varieties vernalised for different periods by supplementing the natural day (April 15 and onwards at Arlington, Va.). In two of these varieties, indeed, the effect of vernalisation was only manifest under continuous light.

With these facts in mind the day-lengths obtaining during the experiment described above may now be examined. The number of hours between sunrise and sunset under which each planting date series began and finished is given in Table X.

TABLE X

*Hours between Sunrise and Sunset at Beginning and End of the Experimental Period for each Planting Date Series*

Planting date:	April 5.	April 19.	May 3.	May 17.
Day-length at planting . . .	13.2	14.1	14.9	15.7
At final examination . . .	14.6	13.8	13.9	12.0

Between the dates given the day-length, of course, rose to a maximum of nearly 17 hours at midsummer.

It is generally understood that a very low light intensity suffices for photo-periodic response. Forster, Tincker, Vasey, and Wadham (1932), assuming that the critical intensity is between 3 and 5 ft. c.p., have estimated that the hours of *effective* daylight exceed those given by rather less than an hour. From their data, however (l.c., Table III), in which the responses of English and Australian spring wheats are compared, it would appear that the three Australian varieties respond to light of this intensity (given as a daily supplement to 10 hours of full daylight) to a much greater degree than do the three English varieties tested. It is doubtful, indeed, if a 'critical' intensity exists. The work of von Denffer (1939) shows that the 'photoperiodic effect', dependent upon mere duration of light and dark periods, cannot be entirely separated from the effects of light intensity. In an 'intermediate' type of barley he showed that after varying degrees of vernalisation *supplementary* light of varying intensity directly affected time to flowering and leaf number in proportion to the intensity. Reduction of light intensity acted in every case in the same way as shortening the day-length would have done, increasing both leaf number and time to anthesis.

Since at the earliest planting date the daily duration of light exceeded 13 hours and increased by 1 hour during the first 2 weeks, and since at least 4 weeks' exposure to days of 10 hours' duration are required to induce flowering in rye, it seems improbable that short-day induction plays any part in producing the observed effects. Short-day induction in late sowings was not possible because it requires subsequent long days for its full expression; in any case the less completely vernalised series were retarded by late sowing. There remains therefore for consideration the accelerating effect of long days; this can only operate when flower initiation has already taken place. After longer periods of vernalisation early sowing might therefore be expected to retard flowering since the plants would reach the stage in development at which long days are optimal at an early date and so at a time of relatively short days. With shorter periods of vernalisation, on the other hand, the stage at which the plant can utilize long days is delayed, so that after late sowing the optimal day-length no longer obtains when this stage is reached. The difference between

the plants grown in the field and those cultured in pots is, of course, quite unrelated to day-length.

(ii) *The effects of temperature during growth.* Von Denffer (1939) found that the effect of sowing date on the flowering of Eckendorffer Mammuth barley was not eliminated when the day-length was artificially controlled. This residual effect he attributed to progressive changes in temperature: possibly increasing light intensity was also concerned. Forster, Tincker, Vasey, and Wadham (1932, Table II) also indicate that temperature as well as day-length contributes to the effect of sowing date on spring wheat grown at Werribee, Australia.

In 1943 the maximum temperatures rose to a high level between April 12 and 19, warming the soil at 4 in. depth, but the accompanying minima were low. After this, persistently low temperatures prevailed until May 10, just before the last sowing date, after which summer conditions were maintained. Thus, the temperature levels at the times of the second and third sowings were uniformly cool and the earliest sown plants actually experienced warmer conditions. Nevertheless, the *number* of cool days occurring after each sowing is diminished by late sowing. In Table XI is given the number of days on which the temperature was above or below values likely to be critical. Similar data for the soil temperatures at 4 in. depth, recorded at 9 a.m., are also given; no data are available for the soil level at which the stem apex of rye grows.

TABLE XI

*Number of Days during the First Eight Weeks after each Sowing Date on which Temperatures above or below Critical Values were recorded*

Sowing date:	April 5.	April 19.	May 3.	May 17.
Soil temperature } 10° C. or less	11	4	3	0
at 4 in. depth } 16° C. or more	13	22	35	47
Air temperatures: Min. = 10° C. or less	51	46	44	40
Max. = 16° C. or more	38	43	48	55

In relation to development, two levels of temperature must be considered, namely, those above the upper limit of effective vernalising temperatures, and the range of natural vernalisation temperature. Once flower primordia are differentiated further progress to flowering under optimal conditions of day-length is largely determined by the rate of extension growth; hence it is accelerated by warmth. Samohina and Ziherman (quoted by Whyte, 1947, p. 146) state that apart from its effect on growth high temperature directly hastens late stages in flower development.

An experiment in 1940 showed the effect of exposure to warm conditions during either the first or the second month after planting vernalised winter rye (Table XII).

Development during the first 4 weeks after planting was significantly retarded by warmth, but this difference was not afterwards maintained in cool conditions. Warmth during the second period of 4 weeks, on the other hand,

TABLE XII

*Progress to Flowering of Winter Rye vernalised 7 Weeks and exposed to Varying Temperature Conditions after Planting*

Temperature during weeks			'Score' at	Difference.	Days to anthesis.		Leaf
1-4.	4-8.	8-end.	4 weeks.		Difference.	number.	
Cool	Cool	Cool	28.4±0.7	3.2±0.9	58.5±0.5	0.2±1.2	8.9±0.2
Warm	Cool	Cool	25.2±0.5		58.3±1.0		8.5±0.4
Cool	Warm	Cool	—		54.3±1.0		8.5±0.2

(Difference between warm and cool, about  $5^{\circ}\text{C.}$ )

accelerated flowering to a small but significant degree. This acceleration was not accompanied by any change in leaf number, which demonstrates that it was due to the increased growth-rate during the shooting stage in development. Warmth immediately following vernalisation had some immediate retarding effect but did not, in this case, delay anthesis.

Both Efeikin (1941) and Tetjurev (1941) found that vernalised winter wheat was completely devernalised by 3 days' treatment at  $35^{\circ}\text{C.}$  On the other hand, in an experiment carried out in 1944 with Petkus winter rye, grain vernalised for 6 weeks was only partially devernalised by exposure for various periods to temperatures ranging from  $25^{\circ}$  to  $40^{\circ}\text{C.}$  (Gregory and Purvis, 1945). After 12 weeks' vernalisation no heat effect was observed at all. This suggested that the extent of devernalisation possible depended on the duration of vernalisation which preceded the heat treatment. The results of further work on this subject will be embodied in a paper now in preparation; the relevant points may, however, be summarized as follows: (a) Spring rye is very little retarded by heat treatment, nor is the 'score' for unvernalsed winter rye reduced. (b) With vernalised winter rye the devernalisation varies inversely with the completeness of the vernalisation treatment, indicating that vernalisation becomes irreversible as it proceeds. (c) With unlimited moisture supply and unimpeded growth during vernalisation the process more rapidly attains the irreversible condition, so that when growth is prevented by limited moisture during vernalisation (Russian technique) devernalisation is greater than when vernalisation has been carried out in moist sand. (d) There is some evidence that after heat devernalisation further cold very quickly restores the vernalised condition; the effect of alternating warm and cold periods of varying duration cannot be predicted. (e) The temperature required for devernalisation is not high; some devernalisation occurs at  $18^{\circ}\text{C.}$

There are, then, two possible effects of high temperatures after planting vernalised seedlings. (1) Before the stage of flower differentiation, a temperature sufficiently high has a direct retarding effect on subsequent flowering, and, of course, presupposes absence of natural vernalisation. Partially vernalised plants are especially liable to this retarding effect and are, moreover, subject to it for a longer period, since in them flower initiation takes place later. The technique of vernalisation employed in this experiment (namely, vernalisation with unlimited growth) tends to minimize the effect of

subsequent warmth. (2) After flower initiation warmth uniformly hastens progress to flowering, thus preferentially accelerating the series vernalised for long periods.

The probability of the occurrence of 'natural vernalisation' depends on two factors: (1) the relation of the temperature level to the rate of the vernalisation process, and (2) the neutralising effect of alternating periods of high temperature, such as occur during the day in spring. Gassner (1918) showed that earliness in winter rye and wheat was in inverse proportion to the temperature during germination (see Kidd and West, 1919, Fig. 2). The author's preliminary experiments on vernalisation gave a similar result.

In the author's early work, however, as in Gassner's, the low-temperature treatments were planned in such a way as to ensure equality of size in the seedlings at planting. To provide this, the treatments at higher temperatures were curtailed and thus the duration of exposure varied inversely as the temperature.

In 1943 winter rye was vernalised at different low temperatures. Treatment was given in sand with unlimited moisture and at each temperature the treatment was applied for three different periods. These periods were, however, shorter for the higher temperatures in order to avoid excessive growth. The seedlings were planted out on May 5, 6, and 7 on the same plot as the 'duration' experiment described above; thus a direct comparison with the results of the third sowing (vernalised at 1° C.) is valid. Seedlings from the same series were also planted in pots. To these were added a number which had been treated for 6 weeks at each of the temperatures in question; some of these were of course very etiolated at the time of planting, but soon recovered their normal colour.

The results are given in Table XIII, which includes for comparison the relevant figures from Tables IV and VIII.

TABLE XIII

*Winter Rye vernalised (Unlimited Moisture) for Different Periods at the Temperatures stated, and planted May 3-7. 'Scores' 133 Days after Planting. Number of Replicates in Brackets (1943)*

Temperature: 1° C.	3° C.	5° C.	7° C.	10° C.	
<i>Field.</i>					
Vernalised.					
2 weeks	31±1.9 (62)	30±2.7 (42)	35±2.6 (67)	36±2.9 (53)	27±1.2 (76)
3 "	44±3.5 (62)	—	62±6.7 (58)	40±3.2 (61)	—
4 "	97±3.5 (64)	95±3.1 (56)	94±3.4 (68)	—	—
6 "	115±2.3 (65)	115±1.0 (78)	—	—	—
<i>Pots.</i>					
2 weeks	55±7.6 (12)	64±6.3 (13)	72±8.0 (13)	70±7.9 (9)	37±6.1 (16)
3 "	89±2.7 (6)	—	96±3.5 (14)	82±4.3 (20)	—
4 "	112±1.8 (15)	107±1.0 (15)	114±0.9 (20)	—	—
6 "	119±0.8 (14)	122±0.6 (20)	117±1.5 (31)	119±0.8 (27)	89±3.2 (22)
Unvernalised controls, field grown 29±1.4 (53); in pots 33±5.1 (13).					

The figures given in Table XIII leave little doubt that vernalisation is equally effective for winter rye at all temperatures from 1° C. to 7° C., while at 10° C. the process is slower, but, nevertheless, extended treatment results in a considerable acceleration of flowering. This experiment was repeated in 1944 using the 'limited moisture' method of vernalisation, so that at the higher temperatures etiolation would not be a complicating factor.

The 'scores' are given in Table XIV.

TABLE XIV

*Winter Rye, vernalised (Russian Technique) for 6 Weeks at Different Temperatures. 'Scores' 133 Days after Planting. Number of Replicates in Brackets (1944)*

Temperature:	1° C.	5° C.	7° C.	10° C.
Field culture	101±3.0 (47)	102±5.8 (22)	95±6.0 (28)	50±7.9 (18)
Pot culture	110±1.2 (27)	111±1.0 (26)	112±1.4 (20)	93±3.6 (14)

Here, in the main, the results obtained in the previous year are confirmed, although the plants grown in the field show a smaller response to vernalisation at 7° C. It appears then that for Petkus winter rye a temperature of 10° C. or less is likely to bring about some vernalisation; at the same time it must be remembered that the daily duration of low-temperature conditions in the spring is not likely to be long, and that when short periods at vernalising temperatures alternate with equal periods at high temperature, the effect of the low temperature is neutralized (Gregory and Purvis, 1938, and in the press). This complete neutralization, however, is only known to occur when the previous vernalisation period is very short. It may be that natural vernalisation of several days' duration, superimposed on applied vernalisation exceeding a minimum duration, is fixed and no longer liable to reversal. This would introduce a further differential effect. Lojkin (1936) found that the minimal effective duration of vernalisation was shortened by early sowing. This she attributed entirely to 'natural' vernalisation. She estimated from weather charts the total period during which the temperature was below 10° C. (the level named critical by Lysenko; cf. also Table XI), and when this was added to the period of applied vernalisation the minimal effective period was little shorter after early than after late sowing. This argument is, however, based on the assumption that the rate of the vernalisation process is constant and the effect proportional to the time of exposure (Lojkin, *ibid.*, Fig. 3); it also assumes that there is no reversal of the process during the warmer periods. It explains therefore the changing response to a particular period of treatment but not the changing form of the curves as seen in Fig. 3 (this paper).

If, however, the process of vernalisation is autocatalytic and thus proceeds at first with increasing velocity, and there is abundant evidence, apart from these sigmoid curves, that it is so (Purvis, 1944, 1947), then for each short period of applied vernalisation a 'natural' supplement will have a different effect. If the 'natural' supplement has an effect on flowering equal to that of the minimal period of low temperature required to produce any substantial

effect in the sigmoid 'duration' curve, then the apparent zero point (with no artificial low temperature) on the derived curve will coincide with the point at which the primary curve is sharply inflected upwards. The 'derived' curve will thus be parallel from its inception with the steepest and straightest part of the 'primary' curve.

'Natural' vernalisation is therefore a factor to be considered in interpreting the curves of Fig. 3 provided that it is not neutralized by high day temperatures.

#### IV. DISCUSSION

##### (a) *Deductions from the sowing-date experiment*

It is obviously of theoretical importance to establish the relation between 'intensity' of vernalisation and the duration of the low-temperature treatment. Unfortunately it is not at present possible to assess the degree of vernalisation of the seed at the end of treatment by any direct means; the criteria which have been described by various writers (Richter, 1934; Bassarskaya, 1934, 1936) are merely qualitative, and the shortening of the first leaf (Thimann and Lane, 1938) has been found by Hatcher (unpublished) not to be proportional to the duration of treatment. The required relation can therefore only be deduced from the flowering behaviour of the plants, i.e. the 'scores' entered in the tables in this paper. It should be stated that the frequency distribution of the 'scores' in a sample of partially vernalised plants is definitely asymmetrical, so that the arithmetic means and the standard errors are to some extent uncertain measures.

The very discrepant curves of progress to flowering shown in Fig. 1 demand elucidation, and as the sowing dates used in the two years differed considerably, a direct examination of the interaction of sowing date with duration of treatment was undertaken. The results in terms of 'scores' are shown graphically in Fig. 3 and the salient features of the curves have already been dealt with. The curves there shown may be regarded as variants of a fundamental 'duration' curve and the probable form of this curve will now be considered. It has already been stated that there is evidence that the vernalisation process is autocatalytic. This hypothesis can be tested by using the well-known expression for an autocatalytic reaction,

$$\log_e \frac{x}{A-x} = k(t-t'),$$

where  $x$  represents the 'score' at time  $t$ ,  $A$  is the maximum 'score' attainable, and  $t'$  the time at which vernalisation is half completed. The value for  $A$  used in the test is the 'score' for spring rye grown at the same time as the vernalised winter rye. If, therefore,  $\log_e \frac{x}{A-x}$  is computed and plotted against duration of treatment, a linear relation should hold. In Fig. 4 the values of this function are entered for each of the various sets  $A'-D$  already presented in Fig. 3. No effort has been made to compute the 'line of closest fit' to the

data; the line drawn joins the value computed for  $x = 24$  which is the lowest mean 'score' obtained with unvernalsed winter rye, and the value for the longest duration of vernalisation, using as  $A$  the corresponding value for spring rye. The departures of the experimental values from the line thus approximate to deviations from the 'autocatalytic' values.

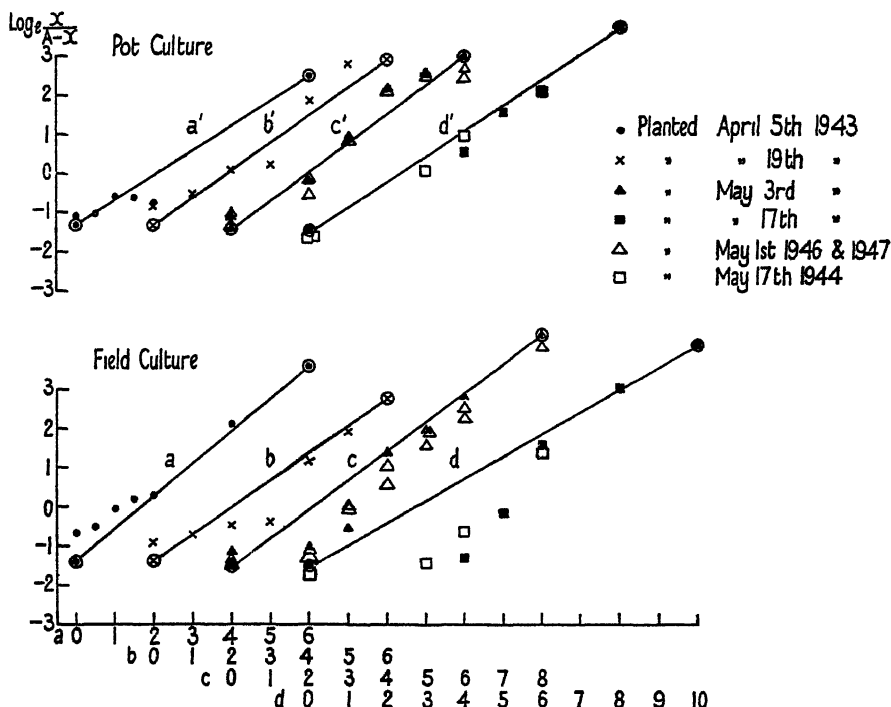


FIG. 4. Effect of planting date on the relation of the value  $\log_e \frac{x}{A-x}$ , where  $x$  = 'score' after known duration of vernalisation,  $A$  = 'score' for spring rye of same planting date (Fig. 3).  $a$ , field culture, and  $a'$ , pot culture, planted April 5;  $b$ , field culture, and  $b'$ , pot culture, planted April 19;  $c$ , field culture, and  $c'$ , pot culture, planted May 3;  $d$ , field culture, and  $d'$ , pot culture, planted May 17. The scales along the abscissa are staggered to show the intervals between the planting dates.

The curves of Fig. 4 ( $a-d$  and  $a'-d'$ ) show that in some sets good agreement obtains with the theoretical values, whereas in others consistent departures appear in both senses. In some of the curves additional points have been added obtained from data in other years. These are distinguished by 'open' symbols corresponding in shape with the 'closed' symbols for the 1943 experiment. These values obtained in different years agree sufficiently well to indicate that the departures from the theoretical values are not in the main due to experimental error. In the last field planting ( $d$ ) the discrepancies are very pronounced, and are not inconsistent with the effects of devernalsation by high temperature, in that the deviations are greatest in partially vernalised

series. At the same time it must be recognized that the retarding effect of progressively shortening days after midsummer would be greater after short periods of vernalisation, since the inception of extension growth, requiring long days, would be delayed in series thus treated.

In the first field planting (*a*) the divergence is without exception positive; there can be little doubt that here 'natural vernalisation' has accelerated flowering in partially vernalised series. It was not expected that the curve *A* in Fig. 3 would conform to the equation for an autocatalytic reaction, for it is itself linear for short durations of vernalisation: the development of a linear from a sigmoid form by summation of the effects of 'natural' and 'artificial' vernalisation has already been discussed (p. 201).

The first planting in pots (*a'*), on the other hand, was protected from 'natural vernalisation' and was, in fact, exposed to somewhat high temperature during early stages prior to May 1; the fact that the distribution of points in this series resembles that in later field plantings may have some significance. In Fig. 3 the 'score' for fully vernalised series and for spring rye is seen to increase with later sowing. In Fig. 4, since the 'score' of spring rye for each sowing date is taken to represent a condition of complete vernalisation, this

increase does not appear; indeed, the value of  $\log_e \frac{x}{A-x}$  for plants grown in

the field after 6 weeks of vernalisation treatment is highest after *early* planting, showing that the effect of 6 weeks of vernalisation approaches the maximum attainable more closely with early planting, although the final 'score' actually increases when planting is delayed by 2 to 4 weeks. This, almost certainly, is a limiting effect of suboptimal day-length during critical stages of the early sown plants in completely vernalised series and in spring rye.

Without direct experimental confirmation of the effects of the environmental factors discussed, the elucidation of the erratic behaviour of partially vernalised plants is largely speculative. The form of the 'duration' curve of vernalisation may be accepted as autocatalytic without much room for doubt; an interesting deduction from this relationship is that vernalisation is half completed in the first 2 weeks of low-temperature treatment.

(b) *The significance of the 'lag' period in the vernalisation of excised embryos*

The resemblance between the effects of late planting and of excision of the embryos on the form of the 'duration' curve is fortuitous and not, in fact, an exact resemblance, for the result of prolonged vernalisation in the two cases is different. The data in Fig. 2 show that the delay due to excision is additional to that due to sowing date and amounts to some 2-4 weeks. The apparent 'lag' period due to late sowing results from retarding processes after planting, while there is considerable evidence that there is, in fact, a delay in the initiation of vernalisation in excised embryos due to the time required to synthesize some substance normally transferred from the endosperm. This time-lag can be shortened by leaving the embryo in contact with the endosperm for a short

time; after 1 week at 1° C. transference of the substance in question appears to be nearly complete. In whole grain this does not necessarily involve a delay of 1 week in the inception of the process, since transfer and utilization of the substance may proceed simultaneously. It is in fact known that vernalisation for as short a period as 4 days accelerates flower development in whole grain. At room temperature the transfer of this substance also begins, but apparently accumulation ceases after 2 days (Table III, p. 188).

The synthesis of this substance and its accumulation by the excised embryo in sufficient quantity to allow vernalisation to proceed at the same rate as in whole grain requires about 2 weeks at low temperature. When the embryos are kept on sucrose medium at 18° C. for 2–4 days before vernalisation treatment, the 'lag' period is not shortened (Table III, p. 188); hence low temperature is requisite either for its synthesis or for its accumulation. This synthesis requires only external sugar-supply and possibly other materials from the embryo itself. It is not inhibited by 4 days' starvation at room temperature, for embryos so treated have been successfully vernalised (Purvis, 1947), but prolonged starvation prevents vernalisation. The nature of the substance synthesized is still unknown; it is, however, certain that it is neither auxin, nor any of the hormone constituents of dried yeast.

#### V. SUMMARY

1. The existence of a 'time-lag' in the vernalisation of excised embryos as compared with whole grain is confirmed by further experiments. After the 'lag' period vernalisation proceeds at the same rate as in whole grain. It is suggested that during the 'lag' period a substance requisite for vernalisation is synthesized, which in whole grain is transferred from the endosperm or aleurone layer.

2. Low temperature is necessary for either the synthesis or the accumulation of this substance in the embryo. Its transference from the endosperm is also facilitated by low temperature. It cannot be replaced by adding hetero-auxin, dried yeast, or ground rye endosperm to the medium on which the embryo germinates.

3. A similar delay in the inception of vernalisation appears to occur when whole grain is planted late in the season (May 17). By means of a sowing-date experiment, it is shown that this delay is entirely due to factors operating *after* planting.

4. The course of vernalisation in whole grain is shown to resemble that of an autocatalytic reaction.

In conclusion the author wishes to thank Prof. F. G. Gregory, F.R.S., for helpful suggestions made at all stages in this work, and especially in the development of the theoretical considerations. The experiments described were carried out at East Malling Research Station, and the vernalisation treatments at Ditton Laboratory; the author wishes to express her gratitude to Dr. R. G. Hatton and Dr. C. West and to the members of their staffs, who by their co-operation have facilitated this work.

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# Dimorphism and Monomorphism in the Plumbaginaceae

## I. A Survey of the Family

BY

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With thirteen Figures in the Text

### INTRODUCTION

VARIATION in the flowers of members of the Plumbaginaceae apparently facilitating cross-pollination was first established by F. Müller (1868), who reported that the flowers of Brazilian species of the genera *Plumbago* and *Limonium* (as *Statice*) were heterostylous. In 1887 Macleod published observations upon flowers of *Limonium vulgare* Mill. (as *Statice Limonium* L.) growing on the Belgian coast. He described the following three varieties (which appear to have been given their names by Knuth, 1899):

- (i) *macrostyla*: style 7–8 mm. long; stigmatic papillae not prominent, occupying 2–2.5 mm. of the style; stamens short; extine of pollen with polygonal markings.
- (ii) *brachystyla*: style 4–5 mm. long; stigmatic papillae small but more prominent; occupying only 0.75–1 mm. of the style; stamens long; extine of pollen with fine grain instead of polygonal markings.
- (iii) *isostyla*: stamens and style about the same length; stigmatic papillae shorter and thicker than in *brachystyla*; pollen as in *brachystyla*.

In addition, he noticed that there were numerous flowers with empty anthers, revealing a tendency to gynodioecism.

Kulczyński (1932) extended the concept of pollen-dimorphism to the genus *Armeria* when dealing with fossil material from Przemyśl, Poland, and correlated stigma-dimorphism was added by Erdtman (1940, 1942), Iversen (1940), and Szafer (1945) for living material. Erdtman (loc. cit.) and Iversen (loc. cit.) re-recorded the dimorphism in *Limonium vulgare*, whilst the latter author showed that pollen-dimorphism was to be found in the genera *Acantholimon* and *Limoniastrum*.

In *Armeria* Iversen demonstrated that the two kinds of plant are completely self-incompatible but cross-compatible. He has also shown that some Arctic and American forms are monomorphic and self-compatible. A comprehensive review of this genus is in the course of preparation as a result of collaboration between Professor G. H. M. Lawrence of the Bailey Hortorium, Ithaca, U.S.A., and the present author. This will be published elsewhere. It is proposed, in subsequent papers of this series, to present the results of

detailed investigations into the genus *Limonium*. However, in view of the fact that some members of the Plumbaginaceae show dimorphism, an investigation of all the genera of the family was considered desirable as a preliminary measure.

#### MATERIAL AND METHODS

Through the kindness of Dr. W. B. Turrill, the author has been permitted to examine a large amount of material from the herbarium of the Royal Botanic Gardens, Kew, Surrey. Investigations have also been made of material from the Herbaria of the British Museum (Natural History), the National Museum of Wales, Cardiff, and the Universities of Manchester and Leeds. The author is extremely grateful to the authorities of these institutions for their willing assistance.

It was possible to remove single flowers or flower-buds from herbarium sheets without damaging the material remaining or obviously altering its appearance. These flowers were mounted whole on slides with 'Durofix' and carried away for examination. The anthers (and styles where possible) were dissected from the flowers for microscopic study. A variety of methods for the rapid investigation of the material was tried, but for general purposes it was found most convenient and perfectly accurate to clear and examine the pollen in xylol. Permanent preparations in balsam could be made directly from interesting specimens. Staining was unnecessary for most purposes. Pollen from fresh material could be examined directly with or without clearing and, if necessary, whole anthers could be dehydrated in alcohol and then cleared in xylol. The anther walls were then removed before adding balsam and mounting.

All genera of the family (as recognized by Pax, 1897) have been examined and the results obtained are summarized in Table I.

Separate polar and equatorial views of the pollen grains are not shown because it was felt that a general view gave a closer approximation to the appearance of the grains under a low-power objective such as would normally be used in routine investigation. The spheroidal or sub-oblate pollen grains of the tribe Plumbagineae are characteristically deeply tricolpate with an ornamentation of rather coarse, blunt spines. No deviation from this condition was encountered and the illustration (Fig. 2) was made from material of *Plumbago europaea* L. The pollen grains of the genus *Aegialitis* (a member of the tribe Staticeae) (Fig. 3) were similar in structure. In the remainder of the Staticeae, however, pollen-dimorphism was encountered in each genus. The illustrations of type A and type B grains which were drawn from *Armeria maritima* (Mill.) Willd. (using the terminology of Iversen (1940)) also represent quite accurately the corresponding types of pollen in the remaining genera.

It is perhaps better to describe first the type B in which the ornamentation of the generally tricolpate grains consists of fine spines disposed more or less at random (Fig. 4) or arranged more obviously in polygons (Fig. 5). The

former extreme approaches more closely to the type of the Plumbagineae, while the latter shows a transition towards the type A grain. Type A grains (Fig. 6) show a complex ornamentation consisting of polygonal areoles surrounded by rods with swollen ends (rounded in surface view, pointed in lateral view). These rods are usually sufficiently closely packed to form

TABLE I  
*Pollen Morphology*

Tribe.	Genus.	Approximate number of species.	Number of species examined as yet.	Types of pollen.
Plumbagineae	Plumbago	20	6	Monomorphic (see Fig. 2)
	Plumbagella	1	1	Monomorphic (structure of grains as in Plumbago)
	Ceratostigma	10	2	Monomorphic (structure of grains as in Plumbago)
	Vogelia	3	2	Monomorphic (structure of grains as in Plumbago)
Staticeae	Aegialitis	2	2	Monomorphic (structure of grains as in Plumbago) (see Fig. 3)
	Limoniastrum	6	2	Dimorphic (types A and B) (structure of grains as in Armeria)
	Limonium	150	40	Dimorphic (types A and B) and secondarily monomorphic (types A and probably B) (structure of grains as in Armeria)
	Goniolimon	10	4	Dimorphic (types A and B) (structure of grains as in Armeria)
	Acantholimon	84	2	Dimorphic (types A and B) (structure of grains as in Armeria)
	Armeria	35	18	Dimorphic (types A and B) and secondarily monomorphic (type A) (see Figs. 4-6)

complete rows. Type A and type B grains are easily distinguishable with low magnification. Associated with type B grains in Limonium and Armeria are the papillate stigmata shown in Fig. 7, while the 'cob' stigmata of Fig. 8 accompany type A grains in dimorphic species and varieties of these genera. In monomorphic Armeria and at least some monomorphic Limonium type A grains are associated with papillate stigmata.

It is apparent immediately that the whole tribe Plumbagineae is characteristically monomorphic and that this monomorphism is of a different kind from that found in Limonium and Armeria. With the exception of Aegialitis,

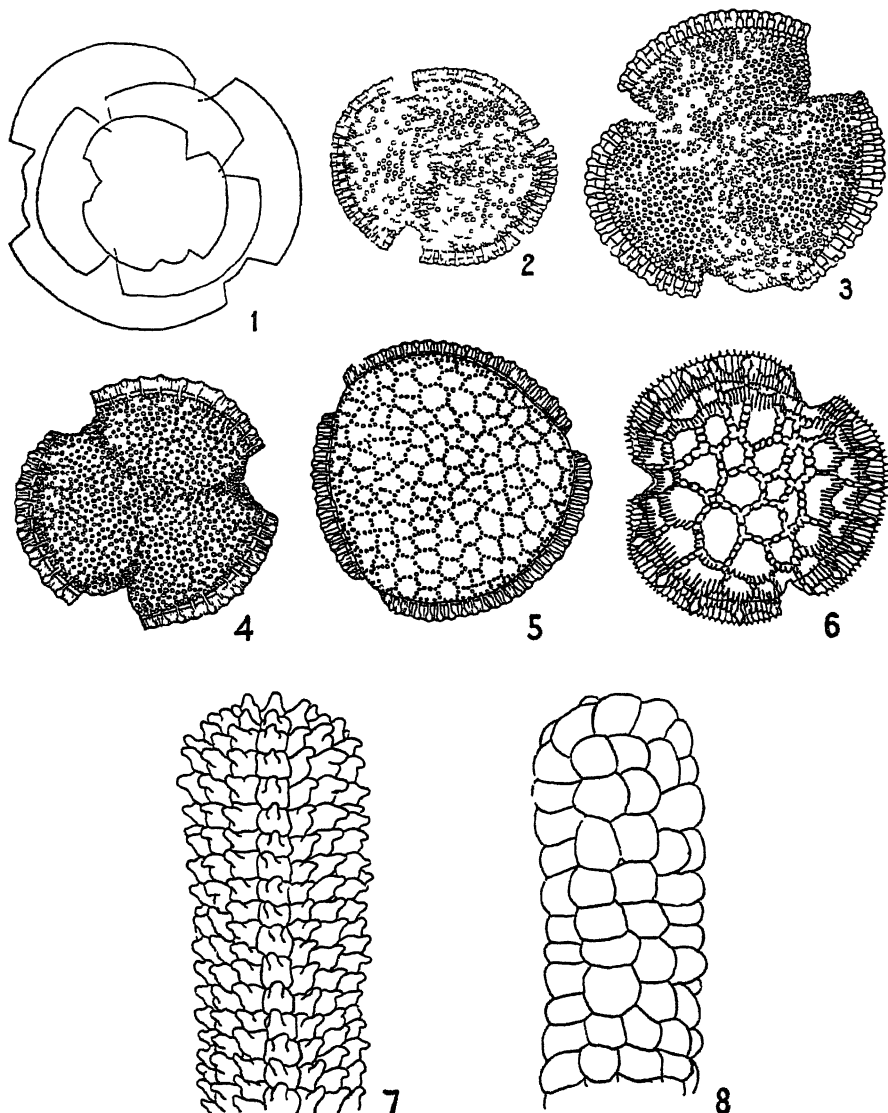


FIG. 1. A comparison of the sizes of pollen grains of *Aegialitis rotundifolia* Robx. (largest), *Ceratostigma plumbaginoides* Bunge (medium), and *Plumbagella micrantha* Spach (smallest). (All  $\times 470$ .) Grains of *Plumbago* and *Vogelia* correspond in size with *Ceratostigma* and *Plumbagella* respectively.

FIG. 2. Pollen grain of *Plumbago europaea* L. in general view. ( $\times 470$ .)

FIG. 3. Pollen grain of *Aegialitis rotundifolia* Robx. in general view. ( $\times 470$ .)

FIG. 4. Pollen grain of type B, *Armeria maritima* (Mill.) Willd. var. *typica* Lawr. (From Woodhall, Wensleydale, Yorkshire.) ( $\times 470$ .)

FIG. 5. Pollen grain of type B, *Armeria maritima*, with spines arranged polygonally (from Jersey, Channel Isles). ( $\times 470$ .)

FIG. 6. Pollen grain of type A, *Armeria maritima* var. *typica* (from Shoreham, Sussex). ( $\times 470$ .)

FIG. 7. Papillate stigma from flower with type B pollen, *Armeria maritima* var. *typica* (from Three Cliffs Bay, Glamorgan). ( $\times 470$ .)

FIG. 8. 'Cob' stigma from flower with type A pollen, *Armeria maritima* var. *typica* (from Shoreham, Sussex). ( $\times 470$ .)

all genera of the tribe Staticeae contain at least some dimorphic species, a fact of great significance in assessing the relationships of these genera.

#### RELATIONS BETWEEN GENERA

##### Tribe Plumbagineae

The distribution of the genus *Plumbago* L. may be classed as pan-tropical (*vide* Maury, 1886). Only *Plumbago europaea* L. and possibly *P. capensis* Thunb. are to be found outside tropical and sub-tropical regions (Fig. 9). *P. zeylanica* L. is distributed throughout southern Asia from Persia to China, in a number of the Pacific islands, the north coast of Australia, and is widespread in Africa (while other species occur in Madagascar and the Mascarene Islands). *P. rosea* L. from south-eastern Asia and the Pacific is also not far removed from *P. zeylanica*, while *P. scandens* L., which occurs in southern North America and Central and South America, is, at least, very closely related. Material has been examined from all the regions covered by this distribution and it is extremely likely that the pollen of this genus is monomorphic throughout. The species of very restricted distribution which have not yet been examined are unlikely to show a different condition. Many of these species are very little differentiated from their wider-ranging neighbours (e.g. *P. Dawei* (Rolfe, 1906) in Uganda and *P. Maximowiczii* (Gandoyer, 1919) in Hawaii are very close to *P. zeylanica*). There cannot be any doubt that the distribution of the genus indicates the considerable age of at least the widespread species and the monomorphism must be considered in relation to this fact.

Dahlgren (1918) has described the heterostyly of three species of this genus. While figuring slight stigma-dimorphism he appears not to have observed any clear distinction between the pollen of the two forms. He reports what seems to be an insignificant difference in pollen-size but did not examine the ornamentation of the grains.

The genus *Plumbagella* Spach consists of a single annual species (*P. micrantha* Spach) which occurs in Tibet and the Altai region (Fig. 10). The structure of its small pollen grains (see Fig. 1) is indistinguishable from that of *Plumbago*, in agreement with its very close relation to that genus. Dahlgren (*loc. cit.*) reported that it is homostylous and self-fertile. *Ceratostigma* Bunge (= *Valoradia* Hochst.) is a small genus to be found in Abyssinia and also from the Himalayas into south-eastern Asia (Fig. 12). It is so closely related to *Plumbago* that Maury (1886) saw no grounds for its separation from that genus. Consequently, the close similarity in pollen morphology is not unexpected (see Fig. 1). *Vogelia* Lam. (= *Dyerophytum* Kuntze) is a relatively distinct genus whose affinities, however, are certainly within this tribe and, once again, the rather small pollen grains (see Fig. 1) resemble those of *Plumbago*. The distributional area of *Vogelia* (Fig. 9) includes South Africa and, also, Socotra, Arabia, and India.

The facts of geographical distribution suggest that this rather well-knit tribe is of considerable antiquity. It would be injudicious for the present

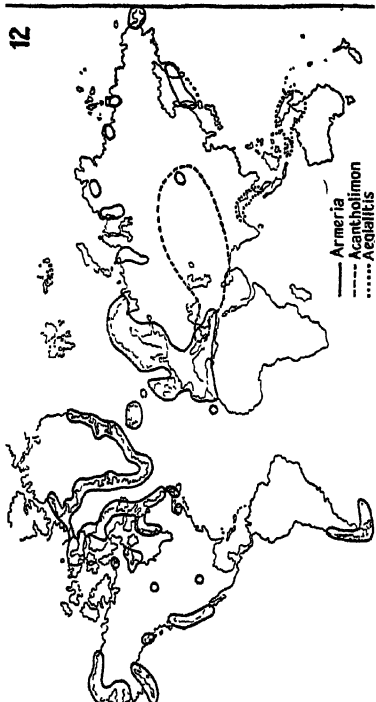
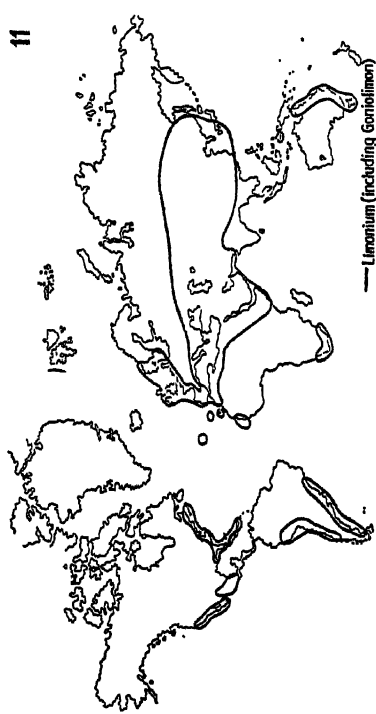
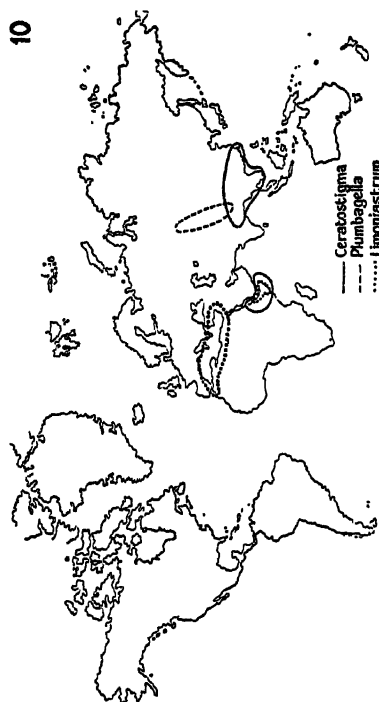
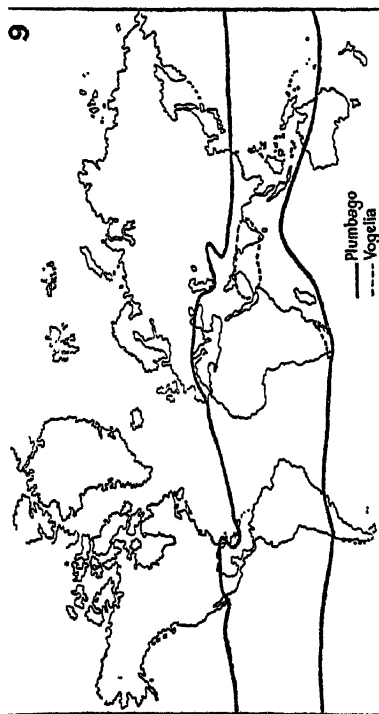


FIG. 9. Approximate northern and southern limits of the pan-tropical genus *Plumbago* (heavy line) and boundaries of the distribution areas of *Vogelia* (broken lines).

FIG. 10. Approximate boundaries of the distribution areas of *Ceratostigma* (continuous lines), *Plumbagella* (broken line), and *Limoniastrum* (dotted line).

FIG. 11. Approximate boundaries of the distribution areas of *Limonium* (including *Gonolimon*).

FIG. 12. Approximate boundaries of the distribution areas of *Armeria* (continuous lines), *Acantholimon* (broken line), and *Aegialitis* (dotted lines).

author to draw conclusions as to the age of the tribe from morphological and anatomical evidence or from the conjectural relations with other families postulated by previous authors. All these have been reported upon in detail by Maury (loc. cit.), and a study of his account has done nothing to change the opinion formed from geography and pollen morphology.

### *Tribe Staticeae*

Generally speaking, this tribe is well differentiated from the Plumbagineae by morphological and anatomical features which are summarized by Boissier (1848), Bentham and Hooker (1873-6), Maury (1886), and Pax (1897). Maury, after mentioning (loc. cit., p. 87) that the family is easily divisible into two tribes on the basis of the habits of the plants, the more or less complete union of the corolla-members, and the modifications in type of inflorescence, remarks that this distinction may be made equally well in regard to anatomy. Nevertheless, there are points of convergence between the two tribes. Thus the same author (loc. cit., p. 92) mentions that a transition towards the Plumbagineae is shown by *Limoniastrum* and also, from the point of view of anatomy, by *Aegialitis*. In stems of the latter genus the presence of a continuous band of fibres in the cortex, the appearance of the highly lignified vascular bundles, and the distribution of sclereides throughout the parenchyma indicate a very distinct structure intermediate between those of *Plumbago* and of *Limonium*.

It is interesting to see how the results of the pollen investigations accord with these suggestions. The identical appearance of both types of pollen in the five dimorphic genera and the linkage with stigma-morphology in *Limonium* and *Armeria* is evidence that dimorphism has probably arisen only once in the tribe. This is especially likely when it is considered that the morphological difference between the types of pollen and stigma is probably without significance in the life-history of the plant and is merely a visible accompaniment of the important incompatibility mechanism. It is not suggested that *Limonium* and *Armeria* will prove to be the only genera to contain derived monomorphic types (for monomorphism has arisen several times within *these* genera) and further investigation may reveal such types in *Limoniastrum*, *Goniolimon*, and *Acantholimon*.

The close relation between these five genera which is shown by pollen morphology is in complete accord with deductions from other considerations. The Asiatic *Goniolimon* Boiss. is so nearly related to *Limonium* Mill. that Bentham and Hooker (1873-6) declined to uphold their separation, and Maury (loc. cit., p. 90) has provided anatomical evidence to support this reunion. In the view of the last author, *Armeria* Willd. and *Acantholimon* Boiss. may demonstrate parallel evolution at opposite ends of the Mediterranean, and there can be little doubt from their geographical distributions (Fig. 12) that they are of more recent origin than the genus *Limonium* (see Fig. 11).

*Limoniastrum* Mönch (= *Bubania* Gir.) is apparently less closely related, but there is no additional evidence from pollen-morphology to add to Maury's

suggestion (reported above) that this genus links the Staticeae with the Plumbagineae. Dimorphism had certainly become established before the evolution of the present-day *Limoniastrum*, which is restricted to the neighbourhood of the Mediterranean Sea with a headquarters in Algeria and Morocco and a possible outlier in Somaliland (Fig. 10).

*Aegialitis* R. Br., however, provides more evidence. A comparison of Figs. 2 and 3 shows the resemblance between the pollen of this genus and that of the Plumbagineae. On the other hand, although, as Maury has suggested, there are anatomical connexions with the Plumbagineae, the same author has been enabled to say (*loc. cit.*, p. 91) that the differences in organography between *Aegialitis* and *Limonium* are feeble. Certainly, this genus does come between the dimorphic genera of the Staticeae and the monomorphic genera of the Plumbagineae and probably developed from the former stock before dimorphism had become established. On this reckoning it should be a more ancient genus than any other within the tribe. The geography of *Aegialitis* (Fig. 12) is not in disagreement with this, for it is the only genus within the Staticeae which is restricted to the Tropics.

*Aegialitis* contains one or possibly two species. This almost monotypic condition in the genus might be considered to be due to extreme age or relative youth according to the evolutionary faith of the interpreter (although anyone favouring the latter interpretation will experience considerable difficulty in finding it an immediate ancestor). *A. annulata* R. Br. grows in the Cape York peninsula of Queensland and the Gulf of Carpentaria, in islands in the Torres Strait, in New Guinea, Timor, and Aru. Maury also maps the occurrence of this species in the North-West Division of Western Australia, but confirmation of this appears to be lacking. The doubtfully distinct *A. rotundifolia* Robx. is found on the Indian and Burmese coasts from Orissa to Mergui and in the Andaman Islands. According to H. N. Ridley (*in litt.*) the record of this species from Malacca attributed to Griffith is doubtful, for this collector's material was very much mixed up when found in cellars formerly belonging to the East India Company. It should be remembered that *Plumbago zeylanica*, which is believed to be ancient, is also found in both Australia and south-eastern Asia. Probably none of the dimorphic genera of the Staticeae have been able to make this passage, although *Limonium billardieri* Kuntze is found on the island of Bouru in the Moluccas. It will be shown in a later paper that the only species of *Limonium* to occur in eastern Australia (*L. australe* Kuntze) has its affinities with species growing in China (*L. sinense* Kuntze) and Japan (*L. japonicum* Kuntze), and its secondary monomorphism may have been of considerable assistance in establishment in Australia after long-distance dispersal.

It appears that the distribution of *Aegialitis* is that of an ancient genus, and the opinion of Maury that the presence of a species of this genus in Australia can only be explained by recent introduction is contested. Nevertheless, it should be recorded that Mr. Ridley believes it to be distributed by sea.

## PHYLOGENY

These studies have given us some idea of the relationships of the genera within the tribes. The connexion of the tribes with each other is more difficult to assess.

Investigations of the origin of the embryo-sac in both sections of the family have shown it to be tetrasporic (cf. Maheshwari, 1946, 1947, 1947a). The subsequent development differs in the two sections. In the Staticeae the

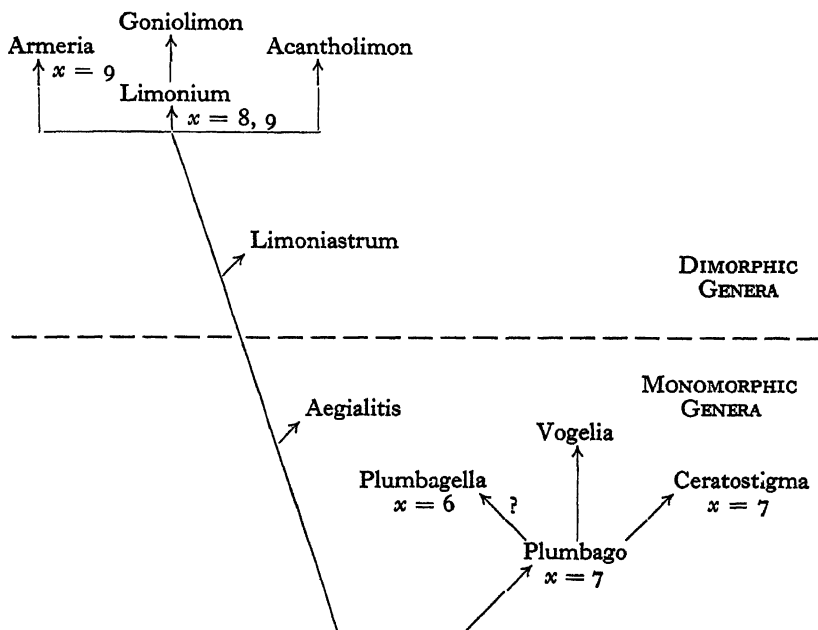


FIG. 13. Phylogenetic scheme for the family Plumbaginaceae.

'Fritillaria' type of development occurs while the genera Plumbago, Ceratostigma, and Vogelia agree in the possession of a very different type which results in the formation of an embryo-sac lacking in synergidae and antipodal cells. Plumbagella displays a unique pattern of development resulting in the formation of a tetranucleate embryo-sac and its phylogenetic position *within* the Plumbagineae needs further investigation. Because of the difference between the tribes it is difficult to derive the Staticeae from the Plumbagineae despite the apparent intermediacy of Aegialitis. The most likely interpretation appears to be that the two tribes are derived from a common ancestor and that Aegialitis, being the most primitive of the Staticeae, has diverged least from the Plumbagineae (Fig. 13). In this figure the basic chromosome numbers for each genus (where these are known) are added. It must be emphasized that our knowledge of the chromosome numbers of this family is very far from complete. Even within the genus Plumbago the count for the most important species (*P. zeylanica*) is unknown as yet.

There is no help to be obtained from the fossil record. Fruiting calyces of *Armeria* have been found in Interglacial and Glacial deposits of the Pleistocene, and this fossil record will be discussed elsewhere. Remains of *Limonium* have been encountered by Swinnerton along with *Armeria* in post-glacial deposits on the Lincolnshire coast (Swinnerton, 1931), but the genera are certainly much older than this. Nevertheless, a large part of the distribution area of *Armeria* may have been attained during the Pleistocene glaciations.

#### THE PROMOTION OF OUT-BREEDING AND IN-BREEDING

The reports by Fritz Müller (1868) and Dahlgren (1918) that species of *Plumbago* show heterostyly is of considerable interest because of the occurrence of a similar condition in *Limonium* where, however, this is linked with pollen- and stigma-dimorphism.

In the present study British and French material of *Limonium vulgare* Mill. have been investigated and it has been found possible to confirm the statement by Macleod (1887) that this species is heterostylous. The plants with 'papillate' stigmata possess type B pollen and the plants with 'cob' stigmata possess type A pollen (see Figs. 7 and 8). The papillate stigmata are borne upon styles which are shorter than, or as long as, the stamens, while the cob stigmata are found on long-styled forms only. Thus there is agreement with Macleod's descriptions except that the stigmata of the homostylous variety are not noticeably intermediate. As the extreme brachystylous form is linked with the homostylous by many intermediates it is doubtful if they should be considered separately. There is some evidence that the homostylic condition is at least as common as the brachystylic in British material of this species, and this may be the explanation of Salmon's remark (1905) that the styles of this species equal or exceed the stamens.

The failure by Knuth (1899) to observe heterostyly in the North Frisian Islands may be due to his material having belonged to *L. humile* Mill. As will be shown in a subsequent paper, this species is secondarily monomorphic and has styles shorter than the stamens.

In *Armeria*, the only other genus to have been investigated satisfactorily, there is no evidence of heterostyly. Iversen (1940) has mentioned this and it is the experience of the present author that the styles are longer than the stamens in the young flower-bud but that the filaments then elongate faster and in the mature flower they may be as long as, or longer than, the styles. There is no evidence of any difference between the two types (A and B) in this respect.

Because the genus *Armeria* shows some evidence of more recent evolution than *Limonium* it might be thought that heterostyly is the primitive method of assisting cross-pollination and that the more efficient incompatibility mechanism made visible by pollen- and stigma-dimorphism has been superimposed upon it in *Limonium* or earlier in the history of the tribe. Heterostyly might then lose its importance and be dropped, giving a condition such

as that seen in *Armeria*. However, the heterostylic *Limonium vulgare* may, in fact, show a relatively advanced condition, for it is a tetraploid species ( $n = 16$  according to Choudhuri, 1942). The only diploid species which it has so far been possible to examine from fresh material is *Limonium sinuatum* Kuntze ( $n = 8$  according to Sugiura, 1936). Cultivated material of this species showed pollen- and stigma-dimorphism, but all flowers of both types were homostylous. It should be remembered that the general chromosome number within *Armeria* is  $n = 9$  (Phillips, 1938, and others), so that the genus is unlikely to have arisen from tetraploid *Limonium*, but might well have arisen from a homostylic diploid. Should this developmental story prove to be correct it will provide a parallel with that of *Primula sinensis*, where Mather and De Winton (1941) have given evidence that the development of the incompatibility mechanism preceded that of heterostyly.

The appearance of occasional male-sterile individuals in both *Limonium* and *Armeria* reveals another potentiality, the development of regular gynodioecism as a means of encouraging out-breeding. On the other hand, the development of secondarily monomorphic types with a single style-length has also occurred and has provided species and varieties whose self-fertility has been of advantage in overcoming distributional barriers. Unfortunately, nothing is yet known of the relative lengths of styles and stamens in other genera of the Staticeae.

Dahlgren (1918) has described heterostyly in *Plumbago rosea*, *P. europaea*, and *P. capensis* in some detail and has suggested that *Ceratostigma* and *Vogelia* are probably heterostylous also, while *Plumbagella* is homostylous. The condition in the last genus may be a derived one. Nevertheless it is likely that heterostyly in the Plumbagineae is not directly related to any within the Staticeae and has resulted from a parallel evolution.

## CONCLUSION

This survey of the Plumbaginaceae has demonstrated that each of the two tribes is characterized by many distinct features of which the difference in pollen morphology is but one. Despite the apparent antiquity of the tribe Plumbagineae there is no evidence that the Staticeae (with a more nearly normal embryo-sac) is derived from it and continual divergence from a remote common ancestor appears to be indicated. It is likely that some of the resemblances between the tribes, of which the development of heterostyly may be quoted as an example, are due to parallel evolution. Pollen-dimorphism appears to have arisen relatively early in the history of the Staticeae and to have survived considerable generic differentiation, probably indicating thereby a positive value in the promotion of out-breeding. Other developments, such as a return to monomorphism with consequent self-compatibility or the establishment of regular gynodioecism, do not appear to have been important in the formation of new genera, but subsequent papers will show their influence *within* the genus *Limonium*.

## SUMMARY

A brief historical summary is given of the discovery of pollen- and stigma-dimorphism within the Plumbaginaceae. A demonstration is given of the need for a complete survey of the whole family as a means of showing the relations between genera and as a prelude to the detailed investigation of separate genera.

Methods for the rapid examination of pollen from herbarium and fresh material are described.

Accounts are given of the structure of the pollen grains of the genera forming the two tribes Plumbagineae and Staticeae. All genera of the former tribe have monomorphic pollen, while all genera of the latter tribe, with the exception of *Aegialitis*, show pollen-dimorphism. The difference between 'type A' and 'type B' pollen in the Staticeae is made clear and the associated stigma-dimorphism is illustrated. Derived monomorphism is shown to occur in *Limonium* and *Armeria* and the associated stigma-morphology is described.

The relationships between *Plumbago*, *Plumbagella*, *Cerastostigma*, and *Vogelia* (forming the Plumbagineae) are discussed. They form a relatively well-knit tribe which, from geographical evidence at least, appears to be of considerable antiquity.

*Aegialitis* is unique among the Staticeae in possessing pollen which agrees in structure with that of the Plumbagineae, and additional evidence is presented to suggest that this genus is the oldest within the tribe and least removed from the Plumbagineae. *Limoniastrum*, which Maury (1886) believed to show connexions with the Plumbagineae, has evolved since the development of the pollen-dimorphism and is probably more recent at least than *Aegialitis*. *Limonium*, *Goniolimon*, *Acantholimon*, and *Armeria* are comparatively closely related.

Maps are given showing the distributions of all the genera of the family.

The data accumulated in the survey are used to provide a phylogenetic scheme. Because of the extreme modification in embryo-sac structure revealed by the Plumbagineae, the Staticeae (which are more nearly normal in this respect) are considered not to be derived directly from the Plumbagineae and both tribes have probably diverged from a relatively remote common ancestor.

The stylar morphology of *Limonium vulgare* is described and, although Macleod's (1887) varieties *macrostyla*, *brachystyla*, and *isostyla* have been found in British and French material, it is doubtful if the last two are fundamentally different.

The development of out-breeding and in-breeding systems is traced as far as the available data allows. Heterostyly in *Limonium* has probably been superimposed upon the incompatibility mechanism made visible by pollen- and stigma-dimorphism. The latter is probably the primitive method of assisting out-breeding within the Staticeae. Derived monomorphism with consequent self-compatibility occurs within *Limonium* and *Armeria* and may

be of value in regions where pollinating insects are sparse and also in facilitating establishment after long-distance dispersal.

The development of heterostyly within the Plumbaginaceae probably represents a parallel evolution and it is not directly related to heterostyly within the Staticeae.

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# Plant Populations

## I. A New Application of Neyman's Contagious Distribution

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With five Figures in the Text

### I. INTRODUCTION

THE application of the quadrat method of botanical analysis to the study of the distribution of species and the estimation of the number of individuals within a given area has hitherto rested largely on the assumption that plants are distributed at random. This assumption, however, is one which, to say the least of it, is displeasing to most plant ecologists, who have not wholly accepted it and who prefer to regard the plant community not so much as a fortuitous dispersion but rather as a patchwork or mosaic of species conditioned by a variety of climatic, edaphic, and biotic factors.

It has been the object of the present investigation to re-examine these different points of view with the intention of seeing how far they are reconcilable on statistical and other grounds. As a result it will be shown that the distribution of at least some of the species in a plant community can be described on the hypothesis that when one individual of a species is present other individuals of the same species are also likely to occur. For example, in cases where a Poisson series will not fit the data a good fit is often obtained with Neyman's contagious series, which means that while we must discard the possibility of the individuals being distributed at random, it is statistically sound to accept the possibility that the data can be described by a heterogeneous distribution in which the individuals tend to occur in clusters or groups. This is not to say that all species are distributed in the same manner, but it appears to bring the statistical approach to the problem of plant distribution more into line with the ideas of the descriptive ecologist.

Blackman (1935) pointed out that in spite of the fundamental importance of the problem the exact distribution of plants within the community had not yet been subject to critical analysis. For his study of some species in grassland associations data was collected by means of a quadrat thrown at random over a specified area within a single type of plant community. The number of individuals of the species occurring in the quadrat was recorded, and where it was not possible to distinguish separate individuals, as in the case of grasses where offshoots arise by vegetative reproduction, the shoots or tillers were counted. In this way the results from a number of quadrats can be assigned to a frequency distribution in which the number of quadrats

with 0, 1, 2, 3, . . . individuals, shoots, or 'units' is tabulated. In order to test for randomness of the distribution a Poisson series is calculated, the values for the successive frequency classes being given by the terms of the series

$$e^{-m}, \quad me^{-m}, \quad \frac{m^2}{2!}e^{-m} \quad \dots \quad \frac{m^x}{x!}e^{-m},$$

where  $m$  is the mean number of units per quadrat and  $e^{-m}$  is the expected number of empty quadrats. The goodness of fit is then tested by means of the  $\chi^2$  test (Fisher, 1946) and the corresponding value of  $P(\chi^2)$  is obtained from the probability tables.  $P(\chi^2) = 0.05$  is taken as the limit of significant deviation and all values of  $P(\chi^2) < 0.05$  show that the deviations from expectation are significant and the observed series cannot be considered to show randomness according to Poisson's Law.

Blackman found that out of 19 species examined in this way only 5 showed the possibility of a Poisson type of distribution. Four of these species were propagated only by seed while the remaining 15 increased by vegetative offshoots as well as by seed. Ashby (1935) worked on populations of *Salicornia europaea* and, using Stevens' (see Appendix, Ashby) test for heterogeneity, came to the conclusion that this species showed a small but significant aggregation of individuals as compared with the hypothesis of randomness. Clapham (1936) used data collected by Steiger from grassland communities of high and low prairie in eastern Nebraska and found that only 4 out of 40 species showed the possibility of being distributed at random. Singh and Chalam (1937) worked on the distribution of certain weeds in cultivated fields left fallow for one or two seasons. They found that species with a high mean density and vegetative reproduction were not distributed at random. Later Singh and Das (1938), confining their attentions to those species in which it was possible to count separate individuals and to lands which had been left fallow for only one year, found that of the 21 species examined 13 showed randomness.

In concluding this brief review of the more recent literature on the subject it is noteworthy that each author in turn remarks on the fact that where the hypothesis of random distribution is not tenable there are too many empty quadrats as compared with the expected distribution according to Poisson's Law.

## II. EXPERIMENTAL RESULTS

### *Method of collecting data*

A quadrat of 500 sq. cm. divided into 25 small quadrats each 20 sq. cm. large was used and the frequency distributions were made up from 100 or 500 contiguous 20-sq. cm. quadrats. If for the present we consider that an appropriate size is one which shows the species to be distributed according to Raunkiaer's frequency classes  $A > B > C \geq D < E$  (Raunkiaer, 1934), where the classes correspond to 5 equal frequency groups ranging from 1 to 100, and where the frequency of a species is the percentage of quadrats

in which it occurs out of the total number of quadrats observed, then the 20-sq. cm. quadrat is found to be a suitable size for all the communities under discussion. For example, in the Limonium Marsh population there were 6 species in class *A*, 2 in *B*, 1 each in *C* and *D*, and 3 in *E*. It may be noted, however, that this is not a very satisfactory criterion for quadrat size, but for lack of better it is quoted here and the subject will be considered further at a later date.

The populations examined in this way include 4 types of maritime communities from the dunes and salt-marshes of Blakeney Point, Norfolk; a maritime community from the reclaimed salt-marsh behind the sea-wall at Havant; and two examples of chalk grassland communities from the North Downs near Otford and the Chilterns near Pitstone.

In both maritime and grassland population there were species which reproduce by seed only and species which reproduce by vegetative offshoots as well as by seed. In each species the individual or the shoot, as it applied, was counted as the statistical unit. Where the total population of an area is given, it is the combined count of the individuals and shoots of all species occurring within the area.

#### *Random distribution*

It is convenient to consider first the *Limonium* Salt-marsh community, details of which are given in Table I. For the present the rare species will be omitted as the small number of frequency classes do not give sufficient number of degrees of freedom for carrying out a  $\chi^2$  test when fitting a Poisson series. These species, however, will be referred to again in a later section on relative variance.

From  $m$ , the mean number of units per quadrat, the corresponding Poisson series was calculated for *Festuca* sp., *Armeria maritima*, *Triglochin maritima*, *Limonium vulgare*, *Plantago maritima*, and *Salicornia stricta* (= *S. europaea*). The  $\chi^2$  test was applied and it will be seen from the probability values in Table I that the hypothesis of random distribution should be discarded for all except *Limonium vulgare*. In each case where a Poisson series gave a bad fit the number of empty quadrats in the observed series was greater than expected, and there was therefore an 'over-dispersion' comparable to that already reported by Blackman, Clapham, and Singh and Das, and confirming the results of Ashby in the case of *Salicornia stricta*.

#### *Neyman's contagious distribution*

It seemed likely that this frequently observed phenomenon, in which there are too many empty quadrats as compared with the expected number according to a Poisson series, could be explained on the basis of heterogeneity as defined by Neyman's contagious distribution.

Neyman (1939) was concerned with describing the distribution of larvae which had recently hatched and started to crawl away from the clusters in which the eggs had been laid. He calls attention to the fact that attempts to fit a Poisson distribution to such data almost invariably failed as in the

observed series there were too many empty plots and too few plots with one larva as compared with the calculated series. He notes that this is a fairly frequent cause of the failure to fit a Poisson series, as, for example, in haemacytometer counts, of which he cites an instance from 'Student's' data on the errors of counting with a haemacytometer. These facts led Neyman to suppose that the distribution might be of the same type as described by Pólya as 'contagious', in that the presence of one individual in a particular region increased the possibility of there being other individuals present. In the case of larvae, if one is found in the experimental plot it is likely that there will be others if the eggs are laid in clusters. Referring the argument to plants we can say, in the case of vegetative propagation, that when there is one offshoot from the original plant it is likely that there will be others. In the case of propagation by seed the method of dispersal would affect the distribution. It might be expected that distribution at random would occur where single seeds are dispersed by wind or water, but in the case of dispersal by animals or birds, particularly where whole fruits containing several seeds are carried away, it is highly probable that the individuals would subsequently occur in clusters. Blackman came to the conclusion that where plants were reproduced by means of short rhizomes, it was not surprising to find that the distribution was not at random and that in fact the system would tend to groups. Considered from this point of view one would expect a strong grouping effect from plants with short rhizomes and a greater tendency to randomness from plants with long rhizomes, but morphological characters of this nature may more advantageously be considered at a later date. It is sufficient to note that the principle of contagion is in agreement with certain observed facts.

In deriving the contagious series Neyman makes two hypotheses: (i) that the centres of clusters of eggs are distributed at random, and (ii) that the number of larvae forming each group is a random variable obeying Poisson's law. Following these assumptions two parameters may be defined:

$m_1$  is proportional to the estimated mean number of clusters per unit area of the field,

$m_2$  is proportional to the mean number of units per cluster;

$m_1$  and  $m_2$  may be obtained from the first and second moments of the distribution where

$$\mu_1 = m_1 m_2 \quad \text{and} \quad \mu_2 = m_1 m_2 (1 + m_2)$$

and therefore

$$m_2 = (\mu_2 - \mu_1) / \mu_1 \quad \text{and} \quad m_1 = \mu_1 / m_2.$$

The successive terms of the series may be calculated from the equations suggested by Beall (1940), the first term being given by

$$P(x = 0) = e^{-m_1(1-e^{-m_2})}$$

and subsequent terms by substituting  $k = 0, 1, 2, 3, \dots$  in the equation

$$P(x = k+1) = \frac{m_1 m_2 e^{-m_2}}{k+1} \sum_{t=0}^k \frac{m_2^t}{t!} P(x = k-t).$$

It may be noted that Neyman suggested that in the derivation of this series some of his assumptions may have tended towards over-simplification and therefore he has also provided, in addition to the original type A distribution, two more complicated distributions, type B and type C, in which the tendencies to over-simplification have been adjusted.

Beall, working on entomological data, compared the three types of distribution and found that where all three fitted equally well they were numerically much the same. He concluded that type A is the most easily calculated and most generally useful of the three. When discrepancies between the types do occur, all cases to which they were fitted showed that as one goes from type A to type B to type C the expectation for class O falls and for classes immediately after O tends to rise and then to fall for all subsequent classes.

It will be seen from Neyman's original hypothesis that the contagious distribution he describes is a generalization of Poisson's law, for in the case where  $m_1$  approaches something large, and  $m_2$  becomes very small, and  $m_1 m_2$  is finite, the individuals can no longer be said to be contagious but are distributed at random.

In applying Neyman's contagious distribution to the analysis of plant populations the type A series with two parameters  $m_1$  and  $m_2$  has been used, and it has been assumed that  $m_1$  is proportional to the mean number of groups per unit area of the field and  $m_2$  to the mean number of shoots or individual plants per group. The procedure, then, is to fit a Poisson series, apply the  $\chi^2$  test and, if the hypothesis of a random distribution of individuals does not seem to be borne out by the data, fit Neyman's series and again use the  $\chi^2$  test. It should be noted that the number of degrees of freedom in the latter case is given by  $f = n - 3$ , where  $n$  is the number of frequency classes.

A contagious series has been fitted to the 5 most frequent species in the Limonium Salt-marsh population. In Table I the parameters  $m_1$  and  $m_2$  are

TABLE I  
*Limonium* Salt-marsh, Blakeney Point

Species.	Percentage frequency.	Parameter for Poisson		Relative variance		Parameters for Neyman		
		(m).	$P(\chi^2)$ .	$v/m$ .	$P(\chi^2)$ .	$m_1$ .	$m_2$ .	$P(\chi^2)$ .
<i>Artemisia maritima</i>	1	0.01	—	0.99	0.97			
<i>Obione portulacoides</i>	3	0.03	—	0.97	0.92			
<i>Spergularia marginata</i>	3	0.03	—	0.97	0.92			
<i>Puccinellia maritima</i>	6	0.13	—	3.63	0.00			
<i>Cochlearia officinalis</i>	9	0.11	—	0.34	0.00			
<i>Festuca</i> sp.	18	0.67	0.00	4.98	0.00			
<i>Aster tripolium</i>	24	0.43	—	2.23	0.00			
<i>Suaeda maritima</i>	40	0.68	—	1.50	0.00			
<i>Armeria maritima</i>	43	1.58	0.00	3.39	0.00	0.6604	2.3924	0.53
<i>Triglochin maritima</i>	61	1.32	0.00	1.50	0.00	2.640	0.500	0.29
<i>Limonium vulgare</i>	85	2.31	0.25	1.147	0.27	15.5127	0.1489	0.22
<i>Plantago maritima</i>	88	5.05	0.00	2.85	0.00	2.7312	1.8490	0.65
<i>Salicornia stricta</i>	96	6.99	0.00	3.90	0.00	2.4069	2.9041	0.01
Total population	100	19.35	0.00	2.54	0.00	12.5403	1.5430	0.56

Plant names are according to the 'Check-list of British Vascular Plants' (Clapham, 1946).

given with the probability  $P(\chi^2)$  for each species and in Figs. 1-4 the data is compared with the calculated frequency distributions both for a Neyman and a Poisson series. For *A. maritima*, *T. maritima*, and *P. maritima*, where

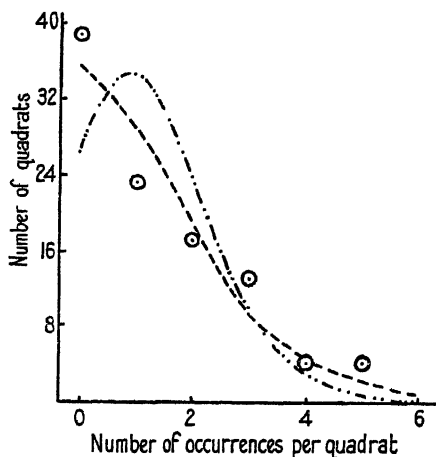


FIG. 1. Frequency distribution of number of shoots per quadrat for *Triglochin maritima* compared with expected frequencies calculated according to a Neyman series ( $P(\chi^2) = 0.29$ ) and a Poisson series ( $P(\chi^2) = 0$ ). Data for figs. 1-5 collected from Limonium Salt-marsh, Blakeney Point, Norfolk, September 1947. For this and subsequent figures Neyman series shown --- and Poisson series - · - ·.

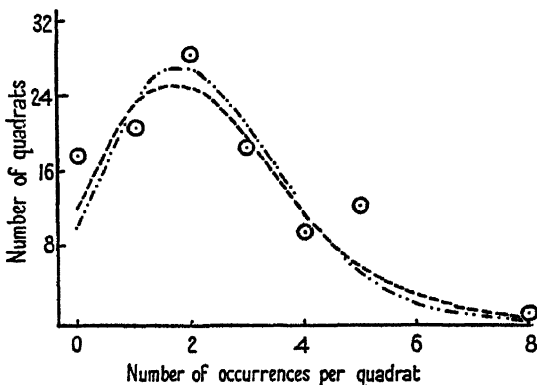


FIG. 2. Frequency distribution for shoots of *Limonium vulgare*.  
Neyman  $P(\chi^2) = 0.22$  and Poisson  $P(\chi^2) = 0.25$ .

the hypothesis of randomness should be discarded, Neyman's series shows a remarkably good fit, for in each case the probability value  $P(\chi^2)$  is greater than 0.25. In the case of *L. vulgare* Neyman's series gives a fairly good fit,  $P(\chi^2) = 0.22$ , but as Fig. 2 clearly shows, a Poisson series seems to give an even better agreement with the observed distribution, which should therefore be accepted as one showing randomness. This species illustrates the theory

of contagion that when  $m_1$  is large (15.5) and  $m_2$  is small (0.15) the distribution can better be considered as being governed by the Poisson Law.

The distribution of the individuals of *S. stricta* is shown in Fig. 4, and it will be seen that a Neyman series certainly gives a better fit than a Poisson

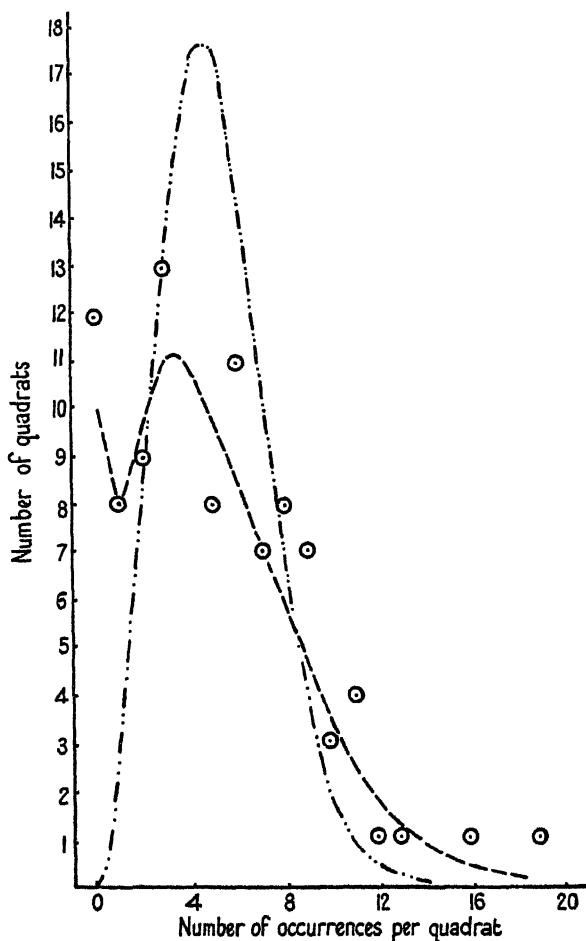


FIG. 3. Frequency distribution for shoots of *Plantago maritima*.  
Neyman  $P(\chi^2) = 0.65$  and Poisson  $P(\chi^2) = 0$ .

series, but even so the probability value  $P(\chi^2) = 0.01$  is less than the significant value  $P(\chi^2) = 0.05$ . However, the general form of the distribution calculated according to Neyman's series agrees with that of the data except that it shows too many empty quadrats and too few quadrats with a small number of individuals. It has already been remarked that Beall found that Neyman's type B and type C distributions showed progressively a smaller number of empty plots and a larger number of plots with fewer individuals,

and therefore it is not unlikely that one of the other types of contagious distribution would show a better fit.

If we assume that individuals and shoots can be treated as equivalent statistical units it is possible to sum the data for all species and so obtain the frequency distribution for the whole population. On fitting a Poisson series we find that the random distribution of units is unlikely, but for the contagious distribution there is an extremely good fit (see Fig. 5,  $P(\chi^2) = 0.5$ ). Therefore

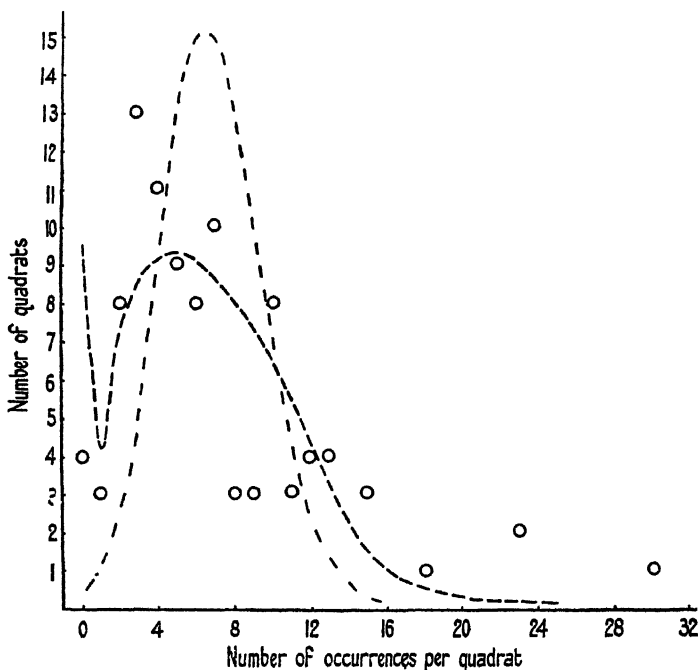


FIG. 4. Frequency distribution for individuals of *Saluorma stricta*.  
Neyman  $P(\chi^2) = 0.01$  and Poisson  $P(\chi^2) = 0$ .

it appears that a population which is the sum of species showing both random and contagious distribution can be described by the contagious series.

### Bimodality

Perhaps one of the most interesting features of Neyman's series is that under certain conditions it gives a distribution with more than one mode. In Table II the data for the distribution of *A. maritima* is compared with the two calculated distributions and it will be seen how very closely Neyman's series reproduces the two modes of the observed distribution. Similar bimodal distributions are shown in Figs. 3 and 4 for *P. maritima* and *S. stricta*. Beall found bimodal curves for the distribution of certain insect populations and Blackman's data for *Primula auricula* also appears to show two modes, whilst some of the distributions given by Clapham, and by Singh and Das, clearly show two modes.

TABLE II  
*Armeria maritima*

Number of shoots per quadrat.	Number of quadrats		
	Observed.	Poisson.	Neyman.
0	57	20.6	54.9
1	6	32.5	7.9
2	12	25.7	10.1
3	5	13.5	9.0
4	5	5.4	6.5
5	5	1.7	4.3
6	7	0.5	2.8
7	1	0.1	1.8
8	—	—	1.1
9	1	—	0.9
10	1	—	0.4
11 and after	—	—	0.3
	100	100.0	100.0

 $P(\chi^2) 0.000$  $P(\chi^2) 0.53$ *The relative variance test for randomness*

It is a property of the Poisson series that the variance ( $v$ ), or square of the standard deviation, is equal to the mean ( $m$ ), so that the ratio variance/mean ( $v/m$ ) is equal to unity for the theoretical series. Clapham used this relative variance ratio as an indication of randomness. But the ratio will vary for observational material and it becomes necessary to decide whether the deviations of the ratio from unity are such as could be attributed to chance or whether they do represent in fact a departure from randomness as expressed by Poisson. A ratio of  $v/m = 1.147$  for *L. vulgare* gave, on fitting a Poisson, a  $P(\chi^2)$  of 0.25, indicating that the deviation from the expected value of unity is such as might be attributed to chance. The variance/mean ratio test is due to Fisher, who showed that  $Nv/m$  is distributed as  $\chi^2$  with  $f = N - 1$  degrees of freedom. As in the examples we have considered  $N$  is large, it is permissible to use an approximation for  $\chi^2$  and to assume that  $\sqrt{(2\chi^2)} - \sqrt{(2f - 1)}$  is normally distributed about zero with unit standard deviation. The tables of the normal curve may therefore be used to judge significance. For example, in the case of *L. vulgare*

$$\frac{Nv}{m} = \frac{100.2.65}{2.31} = 114.7$$

and

$$\sqrt{229.4} - \sqrt{197} = 1.11,$$

and from the tables of the probability integral  $P(\chi^2) = 0.267$ . In Table I the probability figures for fitting a Poisson series and for using the relative variance test are compared and the reliability of the latter test is confirmed. This test can also be used for rare species where the small number of frequency classes do not allow for sufficient number of degrees of freedom for the Poisson ( $\chi^2$ ) test to be applied.

## Further data

The relative variance test can be used to see to what extent randomness occurs in other plant populations. In Table III the relevant data are given

TABLE III  
Relative Variance Test for Randomness

Species.	Species percentage frequency.	Parameter for Poisson ( $m$ ).	Relative variance ( $v/m$ ).	$P(\chi^2)$ .	Community.
<i>Salicornia stricta</i>	100	66.660	9.020	0.00	Salicornia Salt-marsh, Blakeney Point (June)
<i>Suaeda maritima</i>	3.8	0.060	1.035	0.56	
<i>Aster tripolium</i>	1.8	0.018	0.982	0.81	
<i>Obione portulacoides</i>	0.2	0.002	0.998	0.99	
Total population	100	66.740	9.050	0.00	
<i>Salicornia stricta</i>	100	14.210	3.372	0.00	Ditto (September)
<i>Aster tripolium</i>	1	0.020	1.980	0.00	
Total population	100	14.230	3.375	0.00	
<i>Glaux maritima</i>	99.8	5.796	1.107	0.09	Glaux Low, Blakeney Point
<i>Plantago coronopus</i>	45.2	1.310	3.231	0.00	
<i>Carex arenaria</i>	83	2.426	1.651	0.00	Carex Dune, Blakeney Point
<i>Suaeda maritima</i>	2	0.020	0.980	0.97	Juncus Marsh, Havant
<i>Puccinellia maritima</i>	2	0.090	4.910	0.00	
<i>Spergularia marginata</i>	3	0.040	1.460	0.00	
<i>Salicornia stricta</i>	7	0.090	1.350	0.02	
<i>Plantago maritima</i>	25	0.780	4.297	0.00	
<i>Triglochin maritima</i>	25	0.710	3.186	0.00	
<i>Aster tripolium</i>	55	1.370	4.870	0.00	
<i>Glaux maritima</i>	74	1.260	0.883	0.45	
<i>Juncus gerardii</i>	100	10.590	2.098	0.00	
Total population	100	14.940	1.109	0.38	
<i>Polygala</i> sp.	1	0.01	0.99	0.97	Chalk grassland, Otford
<i>Crataegus</i> sp.	1	0.01	0.99	0.97	
<i>Sorbus</i> sp.	1	0.01	0.99	0.97	
<i>Lotus corniculatus</i>	1	0.01	0.99	0.97	
<i>Arenaria</i> sp.	1	0.01	0.99	0.97	
<i>Agrimonia</i> sp.	1	0.01	0.99	0.97	
<i>Prunella vulgaris</i>	2	0.02	0.98	0.97	
<i>Poterium sanguisorba</i>	2	0.02	0.98	0.97	
<i>Dactylis glomerata</i>	3	0.03	0.97	0.95	
<i>Fragaria</i> sp.	4	0.04	0.96	0.86	
<i>Carduus</i> sp.	8	0.09	1.132	0.31	
<i>Leontodon hispidus</i>	9	0.10	1.10	0.40	
<i>Hieracium</i> sp.	23	0.31	1.335	0.02	
<i>Briza media</i>	35	0.61	1.603	0.00	
<i>Carex</i> sp.	47	0.70	1.271	0.00	
<i>Thymus serpyllum</i>	48	0.67	0.987	0.99	
<i>Festuca</i> sp.	82	5.71	4.056	0.00	
<i>Bromus erectus</i>	88	2.61	1.360	0.01	
Total population	100	11.21	2.043	0.00	

TABLE III—(cont.)

Species.	Species percentage frequency.	Parameter for Poisson (m).	Relative variance (v/m).	P( $\chi^2$ ).	Community.
<i>Plantago media</i>	3	0.042	1.642	0.00	Chalk grassland, Pitstone
<i>Helictotrichon pubescens</i>	8.6	0.011	9.886	0.00	
<i>Plantago lanceolata</i>	10	0.130	1.516	0.00	
<i>Ranunculus bulbosus</i>	26.4	0.372	0.509	0.00	
<i>Helictotrichon pratense</i>	29.2	0.658	1.941	0.00	
<i>Briza media</i>	52.6	1.182	2.281	0.00	
<i>Carex flacca</i>	63.6	1.412	1.590	0.00	

for 4 maritime communities and 2 grassland communities. The results, including those for the Limonium Salt-marsh population, are summarized in Table IV, where they are grouped according to Raunkiaer's 5 percentage frequency classes. It will be seen that only 4 species with a percentage frequency higher than 21 per cent. were found to be distributed at random, whereas for rare species the figure is 19 out of 20, but 14 of these were represented by less than 4 plants, and so the very high probability values of  $P(\chi^2)$  should be treated with caution. However, in the case of *Puccinellia maritima* and *Spergularia marginata*, in the Juncus Marsh community, where 2 or more individuals occur in the same quadrat, the test is sufficiently sensitive to pick out the grouping and discard the possibility of randomness.

TABLE IV

## Summary of Data on Randomness for all Species examined

% frequency class.	Species showing possibility of randomness.	Total species.
1-20	19	29
21-40	—	8
41-60	1	6
61-80	1	3
81-100	2	10
	23	56

In the 7 communities examined there are 9 species which occur in more than 1 population. Of these 9, *S. stricta*, *P. maritima*, *T. maritima*, and *B. media* constantly show that they do not follow Poisson's law, while *O. portulacoides* and *Glaux maritima* show the possibility of randomness in both populations in which they occur. On the other hand, *A. tripolium*, *S. media*, and *S. maritima* show both high and low probability values of  $\chi^2$ . A further point that might be mentioned is that none of the 4 species of *Plantago*, *P. coronopus*, *P. maritima*, *P. lanceolata*, and *P. media*, show the possibility of being distributed at random. These 4 species reproduce by short offshoots as well as by seed and the tendency for the individuals to occur in groups is possibly due to the fact that the inflorescences are short, compact spikes which frequently fall to the ground with the seeds still in the capsules. Blackman also found that data from 4 different populations showed that *P. media* was not distributed at random.

*Total populations*

In the area of Juncus Marsh examined there are only 2 species, *Suaeda maritima* and *Glaux maritima*, which show the possibility of being distributed at random; the remaining 7 species show heterogeneity, yet the total population shows a reasonable chance for the hypothesis of random distribution ( $P(\chi^2) = 0.38$ ). It would appear that in spite of the preponderance of species showing heterogeneity, they combine in a complementary manner to give a result which satisfies Poisson's Law.

On the other hand, the area of grassland from the North Downs near Otford contains 18 species of which only 5 show heterogeneity, yet the total population shows a very small probability in the  $\chi^2$  test for randomness.

The Salicornia Marsh population sampled in June shows a very high mean density for *S. stricta* ( $m = 66.66$ ), and a very low density for the remaining 3 species, so that the total population with a mean density of 66.74 bears a very close resemblance to the distribution of *S. stricta*. In September a second count was made a few feet away from the June count. The population had been greatly reduced by competition and the average number of *S. stricta* plants per quadrat was found to be 14.21, again not much different from that of the total population ( $m = 14.23$ ), which like the June count also showed heterogeneity.

In conclusion it may be said that from the data available it appears that there is no simple relation between the distribution of the separate species and the distribution of the total population. It also appears that in the total population heterogeneity is of more frequent occurrence than the scattering of individuals at random.

## III. DISCUSSION

*Type of reproduction*

Attention has already been drawn to the fact that where randomness has been reported it has generally referred to species where reproduction is by seed only or where separate individuals could be counted. In the populations considered here it will be seen that a number of species in which offshoots may occur showed the possibility of being distributed at random (*Aster*, *Obione*, *Spergularia*, *Prunella*, *Carduus*, *Leontodon*, *Poterium*, and *Dactylis*), but in these instances the plants were generally too young to show vegetative reproduction and, being of low frequency and occurring in separate quadrats, there was no tendency to grouping. The cases of *Limonium vulgare* and *Glaux maritima* are exceptional as both species showed randomness and were of high frequency in their respective communities. Both are propagated by seed as well as by vegetative offshoots. *Limonium* has short shoots close to the parent plant but *Glaux* sends out underground stems, which vary in length according to the soil condition, and on these bulbils are formed which do not grow into new plants until the following season when the parent plant has disappeared. This mode of propagation apparently leads to a fair dispersion of the offspring, which do not remain attached to each other or to the parent plant.

It has been noted that species which show heterogeneity are reproduced by seed or by vegetative propagation and by seed. *Salicornia* with only seed production and constant heterogeneity may be given as an example to show that heterogeneity is not due to the method of counting shoots as statistical units but also occurs where individuals can be counted.

In conclusion it may be said that although there is a tendency for distribution at random to occur more frequently in those species where reproduction

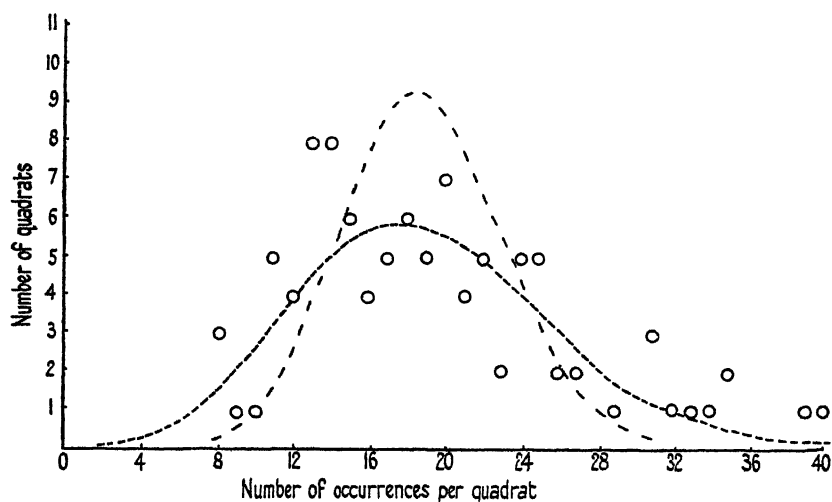


FIG. 5. Frequency distribution of number of occurrences per quadrat for total population of 2,000 sq cm. of *Limonium* Salt-marsh. Neyman  $P(\chi^2) = 0.5$  and Poisson  $P(\chi^2) = 0$ .

is only by seed, there is not yet sufficient evidence for seeking to correlate the type of distribution with the type of reproduction.

### *The principle of contagion*

It has been shown that Neyman's series can be applied to plant populations to test for the hypothesis that when one individual or shoot of a species is present, others of the same species are also likely to occur. Thus it forms a valuable alternative to the Poisson series, which for some time now has dominated the field of the statistical treatment of empirical distributions, and it may explain some of the discrepancies which the biologist has been forced to overlook because they could not be accounted for on the assumption of randomness. Moreover, it is becoming increasingly clear, from the different species that have been analysed in a wide variety of communities, that the distribution of plants at random is much less common than was at first supposed. On the other hand, it has been shown that Neyman's series will give a good fit where a Poisson series fails, particularly in cases where the cause of failure is the excessive number of observations in the first frequency class of the distribution as compared with the number expected according to a Poisson series (see Fig. 5).

In addition Neyman's series is one which may have a wide application if it can be considered to be the generalized form of which the Poisson series is the particular case. The parameter  $m_2$  is proportional to the mean number of units in the group, and when it becomes very small, but  $m_1 m_2$  is finite, we have conditions approximating to a Poisson series. It has been shown that this is the case for *Limonium vulgare*, and a further instance may be quoted for the total population of the Juncus Marsh community where  $m_1 = 136.419$  and  $m_2 = 0.1095$ , and on fitting a Poisson we have  $P(\chi^2) = 0.89$ . It is possible therefore that where the groups overlap and the individuals of one group mingle with the individuals of neighbouring groups, the parameters of Neyman's series can still be used to interpret the population, while it can more particularly be identified by a Poisson series.

Finally it may be said that the principle of contagion is in itself of fundamental importance. For besides being open to statistical treatment it expresses the association of one individual with another in a form that may prove acceptable to the plant ecologist.

#### IV. SUMMARY

The fundamental importance of the distribution of the individuals of plant species is discussed and experimental data from some 10,700 quadrats, based on the populations of small areas of 5 maritime and 2 grassland communities, is given.

It is shown that a new class of 'contagious distribution', originally evolved by J. Neyman for the analysis of insect populations, can be adapted for plant populations, the principle of contagion being that when one individual is present others are also likely to occur.

The series is defined by the parameters  $m_1$  and  $m_2$  which are interpreted for botanical use as

- $m_1$  is proportional to the mean number of groups per unit area of the field,
- $m_2$  is proportional to the mean number of individuals per group.

The first term of the series is given by

$$P(x=0) = e^{-m_1(1-e^{-m_2})}$$

and subsequent terms of the series by

$$P(x=k+1) = \frac{m_1 m_2 e^{-m_2}}{k+1} \sum_{t=0}^k \frac{m_2^t}{t!} P(x=k-t).$$

Where  $m_1$  is large and  $m_2$  is small but  $m_1 m_2$  is finite, the distribution tends towards randomness, so that Neyman's series may be said to be a generalized form of Poisson's series. This being so, the procedure adopted in the analysis of plant populations is to test for randomness, and when the probability value shows that the discrepancy is significant, Neyman's contagious series is fitted.

Six species have been treated in this way and 4 show a high probability of contagious distribution. If it is permissible to treat shoots and individuals

as equivalent statistical units, then all the species of the population may be combined and it is shown for the area of Limonium Marsh under consideration that there is a high probability of contagion.

Previous workers have reported that frequently a Poisson series failed to fit the data because there were too many empty quadrats and too few quadrats with a few individuals. It is shown that Neyman's series gives a good fit in such instances.

One of the most unique features of the contagious series is that it clearly portrays the bimodal nature of the data.

To shorten the test for randomness the relative variance ( $v/m$ ) is used, where

$$\frac{Nv}{m} = \chi^2$$

and  $\sqrt{(2\chi^2)} - \sqrt{(2f-1)}$  is approximately normally distributed.

It is shown that of the 56 species examined, 23 may be distributed at random, and 19 of these are 'rare' species. It is concluded that far from being the general rule it is unusual for a species, particularly a 'frequent' or 'abundant' species, to show randomness.

It is considered that there is not sufficient evidence for attempting to correlate the type of distribution with the type of propagation.

In conclusion it is suggested that this brief survey shows that the application of Neyman's contagious series brings into prominence a very fundamental issue, namely, that biological populations are likely to show contagion or grouping of individuals in a heterogeneous manner rather than a distribution at random.

The author wishes to acknowledge the direction and guidance of Professor W. H. Pearsall, of the Botany Department, University College. She also has much pleasure in thanking Dr. F. N. David, of the Statistics Department, for her advice on the statistical treatment of data and for suggesting that Neyman's series might be used for the analysis of plant populations. The work was carried out with the assistance of a British Council Scholarship.

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# Studies on the Morphology of Anthoceros. I<sup>1</sup>

BY

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With Plate V and nine Figures in the Text

## GENERAL INTRODUCTION

**A**BUNDANT material of *Anthoceros* found in Caernarvonshire showed that a critical study of the taxonomy of the British species of the genus was requisite before a satisfactory identification could be undertaken. It also became apparent that numerous features of the life-cycle had been either incompletely or incorrectly described.

The genus *Anthoceros* was established by Micheli in 1729, that is to say, it is pre-Linnean. It has been one of the most intensively studied archeogoniate plants. The first to describe its life-history in modern terms was Hofmeister (1851), the description forming the opening chapter of his fundamental work on land-plants. This description, though since amended in details, has probably never been surpassed; some parts of it appear to have been forgotten.

During the course of my investigations Dr. P. W. Richards kindly drew my attention to a paper by Rink (1935) on certain East Asiatic Anthocerotaceae. The conclusions he reached about the sex-distribution in one of these species were in full agreement with what I had established for *Anthoceros laevis*. I had also performed identical experiments demonstrating the capacity of the sporophyte to grow in the absence of light, and there is, therefore, no need to describe these.

Large numbers of species of *Anthoceros* have been established without sufficient study of living material; indeed some authors appear to have been familiar with herbarium specimens only. Such specimens are notoriously unsatisfactory for a study of the characteristics of the thallus. The outcome has been a glut of synonyms and inadequately described species, so that Müller in 1940 justifiably speaks of the systematics of *Anthoceros* as one of the darkest chapters in the study of the hepatics. It is hoped that the present work will to some extent contribute to remove this stigma.

The following is a list of the localities from which my material was obtained :

Loc. 1. On a very steep shaded clay precipice down which water was trickling, close to the bank of the river Ogwen, near Llandegai, Caernarvonshire. *A. laevis* and very small amounts of *A. husnoti*, associated with *Pellia fabbromiana*. Observed July–August 1944.

<sup>1</sup> This paper forms part of a thesis for the Ph.D. Degree approved by the University of London.

- Loc. 2. On heavy clay by the side of ditches or waterlogged cart-tracks, frequently shaded by grasses, in a disused quarry, Felin-hên, Caernarvonshire (not far from loc. 1). *A. laevis* and *A. husnoti* associated with *Pellia epiphylla*, *Fossombronia pusilla*, and *Blasia pusilla* (with embryos!). The *A. husnoti* extended at one point on to very rich loam. Observed September–December 1944.
- Loc. 3. *A. laevis* and a little *A. husnoti* supplied by Biological Supply Agency, Aberystwyth, Cardiganshire. January 1945. Detailed locality unknown.
- Loc. 4. The same, from a roadside ditch about a mile inland. January 1945.
- Loc. 5. On moist roadside banks, fallow fields, and the side of a pond near the sea at Beeson, near Kingsbridge, S. Devonshire (Old Red Sandstone). Abundant growth of *A. laevis*, associated with *Fossombronia pusilla*, *F. wondraczeki*, *Pellia epiphylla*, &c. Observed April 1945.
- Loc. 6. Near Looe, Cornwall. *A. laevis*, kindly collected by W. R. and M. E. E. Sherrin. September 1945.
- Loc. 7. On Old Red Sandstone cliffs overlooking the sea at Dawlish, S. Devonshire. *A. laevis* (= *A. 'dichotomus'*) and *A. husnoti* (cf. Macvicar, 1903) associated with *Riccardia multifida*. Some in a sheltered shady situation subjected to a constant trickle of water, the bulk in a very inaccessible position on the side of an overhanging cliff, shaded by grasses. Observed July 1946.
- Loc. 8. On soil of cornfields near Taunton, Somersetshire (New Red Sandstone). *A. 'crispulus'*, with *Riccia glauca*, November 1945, kindly sent by Dr. W. Watson. Also observed in August 1946.
- Loc. 9. Clayey stubble field, near Eridge, Tunbridge Wells, Kent. *A. punctatus*, with *Fossombronia wondraczeki*, *Riccia* sp., *Ephemerum serratum*, &c. Observed September 1946.
- Loc. 10. On loamy soil of a linseed field, Nuneham Courtney, Oxfordshire. Material of *A. punctatus* kindly sent by Mr. A. C. Crundwell, October 1946.
- Loc. 11. The same. From a field at Great Horley, Oxfordshire. November 1946.

The materials from all these localities were cultured separately and used in these investigations. In addition the herbarium materials in the collection of the British Museum (Natural History) and the South London Botanical Institute were examined. Miss E. Armitage very kindly sent me her herbarium sheets of *Anthoceros* for inspection.

My thanks are due to Dr. F. W. Jane who took an interest in these investigations during their early stages, to those who have kindly sent me material, to Mr. W. R. Sherrin for the assistance he gave me in the examination of the museum collections, to Mr. S. Savage who helped me with the

inspection of the Linnean collections, and especially to Professor F. E. Fritsch for helpful discussion and the great trouble he has taken in extending me his advice during the latter part of these investigations.

# I. *ANTHOCEROS LAEVIS* L.

## Introduction

The species of that section of *Anthoceros* to which *A. laevis* belongs are characterized by the absence of mucilage cavities in the tissue of the thallus and by the possession of yellow spores. The only other member of this section stated to occur in Britain is *A. dichotomus* Raddi, collected from cliffs at Dawlish, S. Devonshire (cf. Macvicar, 1903). In the last edition of Macvicar's hepatic flora (1926) the two species are described as agreeing in the characteristics of their sporophytes, the most important difference lying in the shape of the ('sterile') thalli; the measurements are not reliable. The segments of the thalli of *A. dichotomus* are described as dichotomous, channelled, and costate, whereas the thalli of *A. laevis* are given as nearly flat and divided into broad obovate lobes. Both may bear tubers. It was impossible on this basis to allot the abundant material found in various localities to one or other species; the difference in thallus-form just mentioned appeared to lie well within the range of variation.

A close scrutiny of the material, moreover, suggested dioecism and even sexual dimorphism. In most of the relevant literature and in the standard floras (Macvicar, 1926; Müller, 1912-16, 1940) *A. laevis* is described as monoecious. Some authors (e.g. Nicholson, 1911) add that it is markedly protandrous. A search for figures showing male and female organs on the same thallus, such as are usual for *A. punctatus* and its allies, afforded little result. Müller's plate shows a lobe, bearing antheridia, pushing up between the lobes of a thallus with capsules. Cesares-Gil (1919) alone gives a diagram of a thallus bearing capsules in front of antheridial cavities. References to dioecism are to be found in Pearson (1902), while this condition is implied by Micheli (1729) in the description of the new genus *Anthoceros*. His excellent figures of *A. major* (= *A. laevis* L.), showing a rosette of lobes bearing sporophytes and a smaller thallus with antheridial cavities, illustrate clearly the sexual dimorphism.

Among other European forms only *A. dichotomus* is regarded as dioecious, both Müller (1940) and Cesares-Gil (1919) describing it as either monoecious or dioecious, while the latter speaks of evanescent dwarf male lobes. The Dawlish material identified as *A. dichotomus* was originally collected by Blakiston and Tindall, and the latter stated that 'the archegonia are in groups of three in cavities (*sic*!) immediately below the antheridia' (Macvicar, 1903). If this were true, it would represent a unique condition among *Anthocerotales*.

Rink (1935) has demonstrated the occurrence of dioecism and sexual dimorphism on a genetical basis in *Anthocerotaceae* for *Aspiromitus sampalocensis* Burgeff from Luzon.

The cytology of *Anthoceros laevis* has often been studied. In the middle of the last century a lively controversy centred about the distinction between nucleus and pyrenoid (cf. Hofmeister, 1851). The small size of the chromosomes renders investigation difficult. Davis (1899), studying antheridia, sporophyte meristems, and spore mother-cells, and Bagchee (1924), investigating the antheridia of *A. laevis*, found four to be the haploid number. Lorbeer (1924), however, counted eight gemini during meiosis and concluded eight to be the haploid number. Heitz (1927) found four larger and one or two smaller chromosomes in the cells of the thallus of *A. dichotomus*. Tatuno (1934) demonstrated the presence of a heterochromosome in interphase nuclei in *A. laevis* and other Anthocerotaceae. According to him it is the biggest chromosome in a set of six. His drawing of a metaphase plate, however, shows only five chromosomes, including the 'heterochromosome' and a small one. Rink (1935) in *Aspiromitus sampalocensis* found four autosomes and a small sex-chromosome in the haploid sets, the male sex-chromosome being slightly smaller than the female. He concludes that the sex-chromosomes are heterochromatic. The same author also briefly investigated *A. laevis* and gave six as the haploid number of chromosomes; one of his two drawings shows four (possibly five) chromosomes only.

During my own investigations intended to elucidate the problem of sex-distribution in *A. laevis*, some additional facts relating to its sexual reproduction came to light.

### I. *The type*

In the first edition of the 'Species plantarum' (1753) Linné gives *A. laevis* as the second species of *Anthoceros*, with the description 'ANTHOCEROS frondibus indivisis sinuatis laevibus'.<sup>1</sup> He refers to the 'Hortus cliffortianum', the 'Historia muscorum' of Dillen, and the 'Nova plantarum genera' of Micheli. The distribution is given as boreal Europe and America, which is most likely based on Dillen (1740), who gives somewhat diagrammatical illustrations of his 'Anthoceros foliis majoribus, minus laciniatus' drawn from material collected in Germany and Virginia. These figures are labelled *A. laevis* by Linné in his own copy.

Two sheets in the Linnean herbarium are labelled 'Anthoceros' by Linné himself. No. 1272.1 has two samples of material glued to it, one having yellow spores and being annotated in Linné's hand: 'Anthoceros frondibus sinuatis punctatis. Schreber. Anthoceros laevis? Dill. musc. t 68 f 2?' In the Linnean correspondence is a letter from Schreber at Leipzig, dated January 25, 1767, mentioning the dispatch of some *A. laevis*, which is probably this particular material. Sheet 1272.2 is simply labelled 'Anthoceros laevis' (Pl. VI G). It is here considered as the type-sheet. On the sheet are a number of thalli with numerous undehisced capsules and a single dehisced one 2 cm. in length, the latter showing clearly the characteristic

<sup>1</sup> The spelling and definition of the specific name given by Müller (1940) (*laevis* = smooth, with reference to the spores) are thus clearly incorrect (cf. Pl. VI G).

mode of dehiscence by two spirally twisted valves still connected at the apex. Next to this capsule a yellow spore (max. diameter  $42\mu$ ) is embedded in the glue holding the specimen to the paper. The dry thalli were examined under the microscope, but no indication of antheridial cavities was found.

## II. Variation

The variation of *A. laevis* was studied on plants growing (a) in the field; (b) in soil cultures, usually on loam in big porous dishes kept in garden-frames; and (c) in nutrient agar cultures prepared according to the formula recommended by Rink (1935) and using Petri dishes or Erlenmeyer flasks. The results of the various cultures were in full agreement with one another and are summarized below.

A standard habitat may be considered to be one that affords just sufficient moisture at all times of the year to prevent the thalli from drying up, as well as a certain amount of shade from strong sunlight. A young plant, growing under such conditions, will be firmly anchored to the substratum, will possess a somewhat fleshy consistency, and will usually show a liberal infection with *Nostoc*. Branching proceeds along the lines described by Hofmeister (1851), i.e. dichotomy of the growing-points followed only occasionally by deeper forking of the thallus, so that a number of lobes with several growing-points are produced. If sufficient room is available, the plant soon assumes a rosette form. Ultimately the older parts of the thallus at the centre of the rosette decay, the individual lobes thus becoming independent, as in a *Riccia* or *Pellia*. In the formation of the lobes occasional growing-points usually become shifted to their sides and may be left behind in the older regions (cf. Goebel, 1928). These growing-points may later give rise to additional lobes at the centre of the rosette. At first the lobes are flat or slightly concave, but as they become crowded and press against each other, their margins become raised and the individual lobes thus appear broadly channelled (Pl. VI A, B). There is, however, never the development of a distinct mid-rib. If there is sufficient moisture, somewhat narrower channelled lobes usually grow more or less parallel to each other up the steep sides of the culture vessel. Pl. VI c, a patch of the Dawlish material (see § III), growing under comparable conditions up a steep Old Red Sandstone cliff, shows this well.

The lobes of crowded plants ultimately begin to grow obliquely upwards; with their wavy and fleshy margins they give the culture an appearance somewhat reminiscent of the surface of a *Sparassis* (Pl. VI A). During the season of vigorous vegetative activity (summer and autumn) such compact growth may in a culture reach to a height of over 1 cm., the individual lobes affording mutual protection from desiccation. During the winter the plants, now usually bearing their respective sex-organs in abundance, once again grow horizontally and fan out into new lobes. By this time the thallus portions at the original level have decayed and now their independent branches have a shape reminiscent of that of a clawed *Lychnis* petal (Pl. VI D; Text-

fig. 4 E). As consolidation at the new level proceeds the part below continues to decay and a gradual subsidence occurs. The dense carpet, with its abundant rhizoid development, especially from the new horizontal growth, holds water fairly well and is often quite moist when the soil beneath is dry. The same cycle of events is repeated in subsequent years, so that in time the derivatives of an individual plant grow at progressively higher levels by a successive 'step' formation.

In a moister habitat rhizoid development is much reduced and the lobes grow loosely over one another. Their branching becomes irregular, and in general appearance they approach *Riccardia pinguis* (cf. Pl. VI F, plants grown on agar). Nostoc infection is somewhat reduced and the plants are less fertile. In the field this condition is most strikingly seen where plants grow partially submerged at the sides of ditches or where water is constantly trickling over them.

Under low light intensities the plants show a marked phototropic response, which is incidentally very clearly shown by sporelings growing on agar. Thin etiolated upright lobes arise from the margins of the thallus. An extreme example is shown in Pl. VI E. These plants were grown in a crystallizing dish, kept very moist, and illuminated by diffuse daylight from a skylight above them.

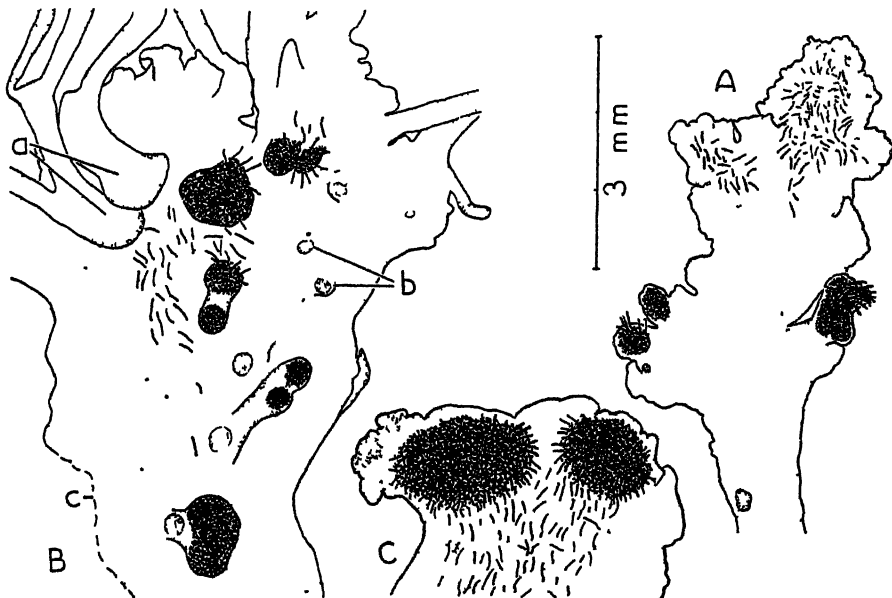
Tuber development in *Anthoceros* has been described by Cesares-Gil (1919), Ch. et R. Duin (1913), and Goebel (1930) amongst others. A thorough search through material in the field or in cultures will generally reveal some tubers and at times they are very abundant. Plants of the 'clawed' type (cf. Pl. VI D) usually show an abundant development of lateral tubers in their older parts in the late winter. These, which are the commonest types of tubers, develop as swellings behind one or more of the growing-points persisting along the sides (Text-fig. 1 A).

As lower regions of a carpet decay, the tubers become isolated. If such growths are broken up in the spring, very large numbers of free tubers are disclosed, most of which have grown out into narrow etiolated upright shoots. They can be compared with the reservoir of young trees beneath the canopy of a forest. Occasionally tubers germinate whilst still in organic connexion with the parent thallus. Tubers are less commonly produced on the ventral surface of the thallus (Text-fig. 1 B). If the growing-point or growing-points, behind which a tuber has been formed, subsequently again take up active growth the tuber becomes ventral; a stalk may or may not be formed as a secondary development. That this is the method of production of these ventral tubers is substantiated by the examination of occasional appropriate specimens. Sprouting of such tubers *in situ* will lead to the production of ventral shoots (Text-fig. 1 B, a). In a few instances a third form of tuber has been found. This consists of a large swelling of the thallus tissue just behind the front margin, and this may occupy the entire width of the lobe (Text-fig. 1 C; a plant from loc. 1). These different types of tubers are connected by intermediate forms.

The structure of the tubers has been studied by Ashworth (1896) on exsiccata of *A. tuberosus* Taylor. My own investigations on the present material are in full agreement with his findings, although a superficial cork layer was not observed. Fungi play no part in tuber formation.

### III. The Dawlish material (locality No. 7)

This was collected at the end of July 1946. The plants found are clearly identical with those described by Macvicar (1903, 1926) and are in agreement



TEXT-FIG. 1. *A. laevis*. Tubers. A. Lateral tubers. B. Ventral tubers (same material as shown in Pl. VI c). a, narrow ventral runners, probably arisen through sprouting of tubers; b, *Nostoc* colonies; c, decaying thallus-margin. c. Subterminal tubers. All from plants bearing archegonia. Seen from lower side Rhizoids shown trimmed.

with his (very scanty) herbarium material. A thorough search was made, and plants in the field were found displaying the whole range of variations described in § II, in accordance with the microclimatic conditions obtaining in different habitats. The most striking form is that shown in Pl. VI c. I have described above how this can be obtained experimentally in cultures of other material. There is no distinct costa. Tubers were present in considerable abundance on irregularly branched plants growing in loose patches with a constant trickle of water over them, as well as on others at the other climatic extreme. The broad subterminal type of tuber (Text-fig. 1 c) was most prominent, but other forms were also present. A few small plants bearing antheridia were seen, while sporophytes were abundant. The characteristics of the latter tally completely with those of the material of *A. laevis* from the other localities. For example, the length of the dehiscing capsules

was 0.7–6.5 cm., in the remaining material 0.8–9 cm. (average 3–4 cm.); spores yellow with short papillae on all faces, longest on the free face, max. diameter 33–47  $\mu$ , in the remaining material 30–47  $\mu$  (average 40  $\mu$ ).

The plants were cultured on their original soil, on garden soil similar to that used for the remainder of the cultures, and on nutrient agar. They soon began to agree closely in general appearance with the plants in corresponding cultures of other material. The tubers became less abundant, the broad ones disappeared mainly through gradual discharge of their contents during the sprouting of the lobes in which they were situated. There seems, however, to be a somewhat greater tendency in the Dawlish strain for the production of broad subterminal tubers, since they appeared again in the soil cultures after a year's growth, although in smaller numbers. In addition plants grown from spores on nutrient agar have several times been noted to produce these tubers as the medium reached exhaustion.

Material from loc. 4 grown on Dawlish soil has the appearance of normal *A. laevis*. It was thought possible that sea-water spray at Dawlish might influence tuber development. During the first 8 months of 1947 plants from localities 4 and 7, growing on ordinary soil, were periodically sprayed with sea-water from an atomizer, but they showed no unusual development.

Howe (1899) examined specimens of *A. dichotomus* Raddi in the Raddi herbarium. He found the spores to be almost wholly smooth on all faces and 42–60  $\mu$  in max. diameter. This is in agreement with my observations made on herbarium material from Rhodia, Crete (*legit* W. E. Nicholson, May 2, 1904), and the 'Archipelago Hetruriae, in insula Montecristo' (E. Levier, *Bryotheca italica*; *legit* S. Sommier, May 6, 1898). The most striking characteristic, apart from the large smooth spores, is the presence of tubers arising from a more or less distinct midrib of the narrow lobes by long (3–4 mm. in the Cretan plant) stalks (cf. diagram in Goebel, 1930).

#### IV. *The distribution of sexes*

Investigation of very large quantities of material from various localities in the field and in cultures, some of which have been under continuous observation for over 3 years, has not led to the discovery of a single specimen bearing both types of sex-organs. Mature antheridia are readily recognized owing to their intense orange colour, but even young antheridial cavities are easily seen as bulges on the thallus. Archegonia only become apparent to the naked eye some time after fertilization. In the monoecious black-spored species a few antheridia will generally be present even on plants bearing sporophytes.

In September 1945 a number of plants bearing antheridia (from loc. 4) were isolated in a culture dish. In November 1947 their derivatives were still growing vigorously and producing the antheridia for the coming season. No lobes bearing archegonia or sporophytes have appeared in the culture.

Plants resulting from spores sown on soil may commence the development of sex-organs while they are still small and young. Usually antheridia appear

first, about half the plants remaining sterile. These later on produce archeogonia directly. (Cf. Text-fig. 4 A, B, and D, 6-months-old sporelings.)

These diverse data suggest that the material is dioecious. There is no evidence that monoecism, even with marked protandry, occurs.

## V. Cytology

(a) *Method.* During the examination of more than 200 preparations of serial sections of *Anthoceros*, stages in nuclear division were very rarely observed. These did not admit of determination of the form of the chromosomes, nor even their exact number, owing to the small size of the chromosomes and their random orientation. In seeking for material which would provide stages in cell-division, the thallus growing-point, the developing antheridia, and the meristematic base and spore mother-cells of the sporophyte seemed most promising. Developing antheridia are satisfactory even when fixed during the day-time (at least in late autumn and early winter), especially as whole sectors of the spermatogenous tissue show synchronized divisions. The tissue of the growing-points of ordinary material was unsatisfactory even when fixed in the early hours of the morning. The method employed by Lorbeer (1934) for other liverworts proved more suitable. Portions of thalli were planted out on nutrient agar or on soil in Petri dishes and subjected to weak daylight, which induced the production of narrow upright lobes. After a week or more the cultures were, prior to fixation, placed in a thermostat in the dark for 3–8 hours; during the winter a temperature of 18–22° C. was suitable, during the warmer months temperatures up to 30° C. were used. Sporophytes, still attached to mother thalli, were placed in moist chambers and transferred directly to the thermostat maintained at the temperature just mentioned.

As already stated, sectioning is not very satisfactory. Direct squashing in combined stain-fixative was tried. Acetic orcein stained mainly the chloroplasts. Aceto-carmin, used with or without short heating, may take over a week to produce proper staining. The technique finally used consisted of fixation in acetic alcohol (1 part glacial ethanoic acid to 3 parts industrial methylated spirit) with or without application of heat; the material was ready for use as soon as the green colour had disappeared from it, although it could be left in the fixative for several weeks without harm. Prior to staining it was transferred to water and then mordanted for about a minute in a 4 per cent. aqueous solution of ferric ammonium alum, a method for which I am indebted to Dr. M. B. Godward, and which will be published by her shortly.<sup>1</sup> After washing, the material was finally transferred to aceto-carmin. The staler the stain is and the more often it has been used, the better and faster it appears to act; the time for the material to reach a satisfactory blackish-purple colour varied from 30 minutes to several hours.

Suitable portions for the preparation of squashes were teased out with needles and mounted in the staining solution. After warming, a sudden sharp

<sup>1</sup> Cf. *Nature*, cvxi (1948), p. 202.

pressure applied to the cover-slip by a finger covered with a thin cloth proved to be the most satisfactory way of liberating the protoplasts, causing them to spread and adhere to the glass. Warming tended to clear the cytoplasm somewhat; if further clearing was necessary it could be brought about by differentiation with 50 per cent. aqueous ethanoic acid. To make permanent preparations the slides were inverted in 10 per cent. ethanoic acid and the cover-slips with adhering squashes floated off. After dehydration the squashes were remounted using Gurr's 'White Xam' medium. Orange-G in clove oil was used as a cytoplasmic counter-stain in some preparations.

(b) *Results.* Observations on stages in cell-division in stained thallus lobes, not subjected to teasing or squashing, were in full agreement with the descriptions given for *A. laevis* (sections) by Lander (1935), and for living cells of *A. punctatus* by Heitz (1942).

A representative series of drawings and photomicrographs of stages in nuclear division taken from all phases of the life-cycle is given in Text-fig. 2 and Pl. VI H-N. In cells of plants bearing antheridia (Text-fig. 2 ♂), at the height of the mitotic prophase (P) there are apparent a nucleolus, four larger chromosomes, and one very small one. One of the four larger chromosomes is invariably attached to the nucleolus for part of its length and must, therefore, bear a nucleolus organizer. The small chromosome is precocious as compared with the four larger ones in so far as, at this stage, it invariably stains more deeply than these. Towards the end of prophase and the beginning of metaphase (P/M) considerable condensation takes place in the larger chromosomes, until they stain as deeply as the small one. At the same time the nucleolus becomes progressively smaller and may become detached from its organizer-chromosome prior to complete disappearance. Metaphase plates are shown in M. Individual identification of the four larger chromosomes is hardly possible, since their shape varies considerably even in neighbouring cells of a squash of spermatogenous tissue, no doubt due to the effects of squashing; their absolute dimensions are, of course, very small. Metaphases, as opposed to other stages, appear with unexpected frequency in antheridia even during the day-time.

Stages from growing-point cells of thalli bearing either archegonia or developing sporophytes (Text-fig. 2 ♀) are similar. The small precocious chromosome is, however, here relatively larger than in plants bearing antheridia.

A comparison of the chromosome sets from plants bearing male or female reproductive organs respectively thus suggests that the haploid set comprises four autosomes, one of which is the 'nucleolus organizer' chromosome, and a small precocious sex-chromosome, of larger volume in the female than in the male. This is borne out very clearly by diploid cells from the meristematic region of the sporophyte (Text-fig. 2 2n). These cells normally contain but a single nucleolus, to which in prophase two chromosomes are attached (P). Occasionally, however, two nucleoli are present, no doubt owing to a failure of their rudiments to fuse during the preceding telophase. In

addition to the eight autosomes there are the two (precocious) sex-chromosomes. Their difference in size is quite evident. M shows a diploid metaphase plate and A an early anaphase.



TEXT-FIG. 2. *A. laevis*. Chromosome sets (from squashes). ♂ = male sets (M from antheridia, the two others from thallus apices). ♀ = female sets (from thallus apices). 2n, diploid sets (from sporophyte-meristem); R!, reduction-division (spore mother cells); P, prophase; M, metaphase; A, anaphase. From cultures of material originating from localities 6, 4, 7, 4; 7, 7, 6, 6; 6, 6, 7; 6, 1, 7.

Although abundant meiotic prophase stages were observed in the examination of spore mother cells, they were not sufficiently clear to admit of unambiguous interpretation. Five much-condensed gemini are seen at the first metaphase (Text-fig. 2 R!, 1. M), the pair representing the sex-chromosomes being recognizable by its smaller size. At this stage it is usually difficult to

distinguish between the male and female sex-chromosomes. Examination of stages from the second meiotic division demonstrates clearly that segregation has taken place. 2. A shows early anaphases of the synchronized divisions of the products of the first meiotic division lying within the common protoplasm of the spore mother cell. That on the right contains the smaller male sex-chromosome.

In interphase nuclei a frequently V-shaped, darkly staining structure is usually apparent by the side of the nucleolus. This structure no doubt represents the 'heterochromosome' of Tatuno (1934). It does not persist as such during nuclear division in the way shown by Tatuno. According to Rink (1935) the sex-chromosome and the ends of two of the autosomes of *Aspiromitus sampalocensis* are heterochromatic. The impression gained during the present study of early prophase is that the 'heterochromosome' is a composite structure formed by an association of various heterochromatic portions; no definite conclusion was, however, reached.

All the above results apply equally to the material from Dawlish and from the other localities (cf. Text-fig. 2).

#### VI. *The culture of isolated spore-tetrads*

It is possible, with the use of fine needles, to isolate individual spore-tetrads and to separate the four constituent spores. Isolated spore-tetrads were planted on sterilized soil in Petri dishes in the summer of 1946. Unfortunately the individual spores were planted too closely together so that it was not possible to recognize with certainty the limits of the resulting plants when they started to bear sex-organs in the subsequent autumn. It was clear, however, that plants with antheridia and with archegonia were present in equal numbers. Further experiments are in progress.<sup>1</sup>

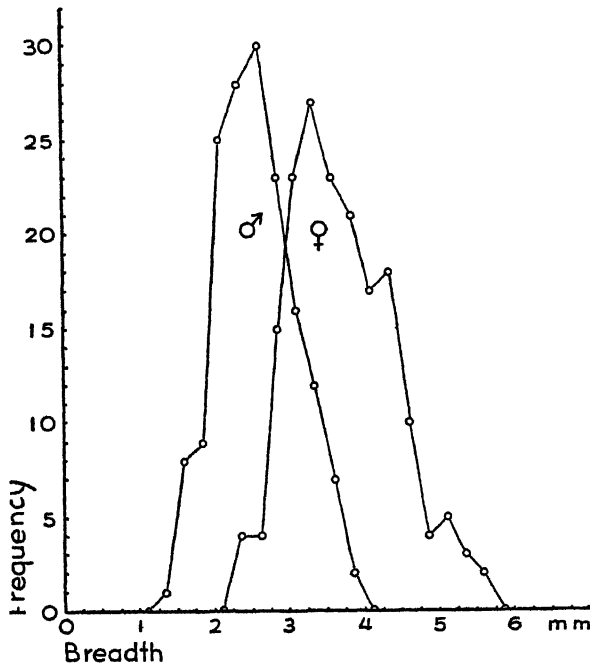
#### VII. *Sexual dimorphism*

Superficial inspection of male and female plants growing under similar conditions shows that the former generally have narrower lobes than the latter. In making such comparisons it must, of course, be borne in mind that the species is very plastic under varying external conditions (cf. § II), but the response is of a similar type for plants of both sexes; cf. Pl. VI E, where the two lower plants bear antheridia.

As the growth of the plants is usually rather irregular, it is difficult to find a suitable characteristic that can be measured for statistical analysis. Rink (1935) measured the breadth of the lobes 'at the point of the dichotomous forking of the thallus' during his investigation of *Aspiromitus sampalocensis*. Owing to the incomplete separation of tissues derived from adjacent growing-points, which is characteristic of Anthocerotaceae, one cannot in ordinary material, as opposed to very etiolated plants, speak of dichotomous forking in the same way as it occurs, say, in a Metzgeria. Where the thallus actually forks, each branch normally already has several apical notches.

<sup>1</sup> It has since been shown that individual spore-tetrads give rise to two male and two female plants.

The variation curves given here (Text-fig. 3) were obtained at the end of December from material growing under crowded conditions in a fairly moist culture (loc. 6) and producing lobes slanting upwards (cf. § II). The breadth of these lobes was measured at the level of a forking in male and female plants respectively. The mean value for the plants with antheridia was 2.6 mm.,



TEXT-FIG. 3. *A. laevis*. Measurements of thallus-lobes of plants bearing antheridia and archegonia respectively.

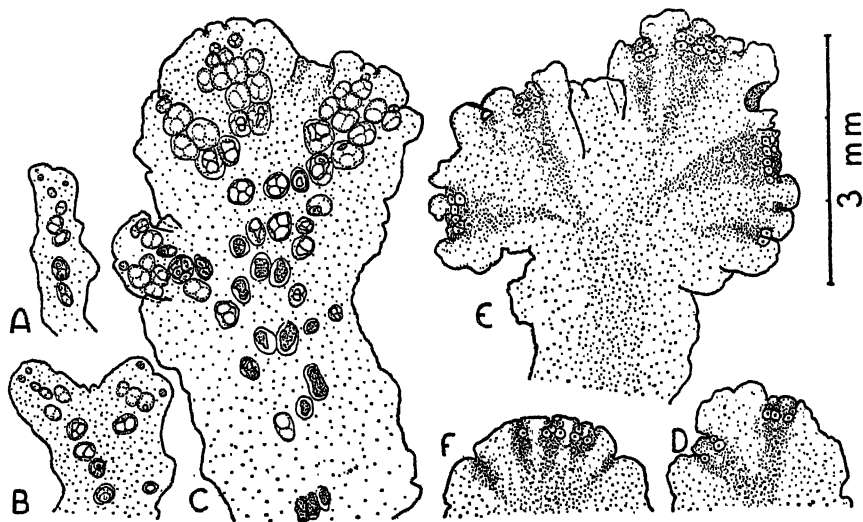
for those with archegonia 3.7 mm. The dimorphism is also plainly recognizable in plants grown on agar. This is evident in Pl. VI F, which shows the products of regeneration of thallus-lobes of male and female plants.

One result of the size difference between fertile plants is the tendency for female to crowd out male plants when they grow intermixed. This is specially striking in cultures, which in consequence, in successive years, show a decrease in the production of sporophytes. Under natural conditions the partial dying away of patches and marginal expansion will provide possibilities for new plants of either sex to develop from spores or tubers and for the more or less unhindered spread of others. During the summer, when as a rule no antheridia are produced, the lobes of the male plants may broaden, while when production of antheridia recommences the lobes gradually narrow by repeated division.

#### VIII. *Observations on the sex-organs*

(a) *Arrangement.* The arrangement on their respective thalli of the antheridial cavities and of the archegonia shows considerable similarities. Both

types of sex-organs are initiated just behind the growing-points, and in sections differentiation is seen to commence very close to the apical cells proper. Each individual growing-point gives rise to a continuous row of sex-organs. This is especially clear in lobes of young sporelings (Text-fig. 4 A, D). In older plants where the growing-points usually lie close together it is usually still possible to trace the series of sex-organs formed from a particular growing-point although there is a certain amount of displacement due to mutual crowding. In this way it is possible to reconstruct the progressive



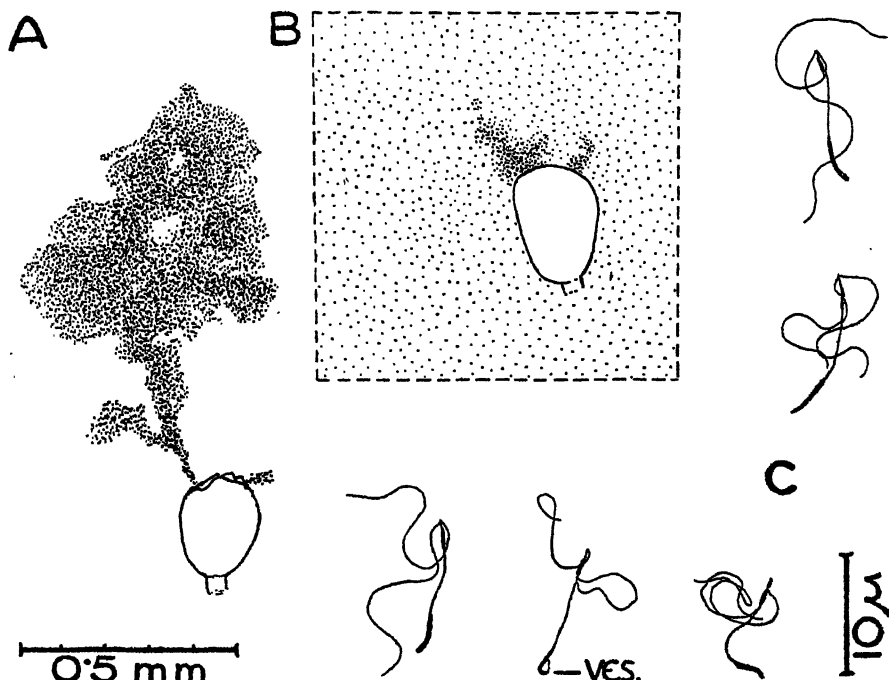
TEXT-FIG. 4. *A. laevis*. Arrangement of antheridial cavities (A-C) and archegonia (D-F). A, B, and D from 6-months-old sporelings.

dichotomies of the growing-point which have led to the momentary form of the thallus. Organs derived from neighbouring apices at more or less the same time tend to form rows parallel to the front margin of the thallus (Text-fig. 4 C, E). As the thallus-lobe elongates, a row of such sex-organs of the same age and level of differentiation becomes separated from rows in front and behind by development of the sterile tissue between. In male plants, where differentiation of sex-organs is prolonged and prominent, this leads to their characteristic arrangement across the thallus in 'bars' visible to the naked eye (C; cf. Pl. VI F, bottom). Archegonial development is usually more restricted; moreover, aborting older archegonia merge with the thallus. They can, however, be traced back in specimens cleared in lactophenol, and their arrangement is then seen to correspond to that of the antheridial cavities (F, where the older archegonia are indicated by thick dots). Whereas lobes bearing antheridial cavities are generally flat or only slightly concave, those bearing archegonia show a deep channelling behind the apical notches, giving them a wavy appearance (cf. Text-fig. 4 A-C with D-F).

(b) *Antheridia and their products*. Two to four antheridia are usually pro-

duced in each antheridial cavity (Text-fig. 4 A-C), although occasionally only a single one or more than four are formed. Their structure has been repeatedly described. Hofmeister (1851) found that the spermatocytes liberated from the antheridia disperse in the surrounding liquid before giving rise to sperms. Muggoch and Walton (1942) state that they had insufficient suitable material for a thorough investigation of spermatocyte discharge in *Anthoceros*.

Dehiscence can be observed if thalli with ripe antheridia are transferred from a fairly dry culture to a drop of water on a slide, or when mature



TEXT-FIG. 5. *A. laevis*. A, B. Opening antheridia extruding spermatocytes (see text). C. Sperms. VES. = Vesicle of residual cytoplasm. Fixed with osmic acid vapour, some stained with Janus green, others dried to the slide and stained in gentian violet.

antheridia are removed with a needle from their cavities and mounted in water. The antheridium absorbs water and an apical aperture arises in the wall by irregular separation of the cells. The mode of subsequent discharge of the contents depends on the degree of maturity. Sometimes the swelling contents squeeze out of the aperture within a few minutes as an opaque mass which even after an hour may not undergo further change. As a rule, however, the aggregate of spermatocytes begins to break up by the violent separation from the periphery of small clusters or of individual spermatocytes, which rise to the surface of the water. In the case of the spermatocyte cloud shown in Text-fig. 5 A, this stage was reached only 45 minutes after its escape, while in that shown in Text-fig. 5 B, where the cloud emerged after the antheridium had been in water for 6 minutes, it dispersed after the lapse of another 4.

At the air-water interface the spermatocytes spread out rapidly, forming a regular pattern, as pointed out by Muggoch and Walton, who concluded that spreading was due to fat demonstrable in the spermatocyte mass. Very ripe antheridia discharge spermatocytes directly in a rapid stream from the aperture. In such a case the liberation of energy results in a rapid backward movement of the isolated antheridia through the water.

The change from spermatocytes into sperms usually occupies some 10 to 20 minutes after spreading has occurred, though sometimes sperms may be liberated at the surface of the cloud, and in certain other cases their production could not be observed. Bagchee's (1924) figure of a sperm is not in agreement with my observations made on living material as well as material fixed by osmic acid vapour (Text-fig. 5 c). The body of the sperm to which the two flagella are attached usually shows some degree of residual curvature, while the usual remnant of spermatocyte cytoplasm adheres for some time to the swollen terminal portion (Text-fig. 5 c, ves.). Just behind the front end and beneath the flagella there is an elongated swelling, probably a blepharoplast.

(c) *Archegonia*. Archegonia are produced and reach maturity close to the actual growing-points, behind which they lie in a slight depression laterally bounded by shoulders of thallus tissue (Text-fig. 4 D-F). Their structure has been frequently described since the time of Hofmeister, although most authors have been mainly concerned with comparative studies. It is surprising that no one mentions the most conspicuous external feature of the mature archegonium, its surrounding mound of mucilage. Janczewski (1872) possibly saw it, but seems to have considered it abnormal. Text-fig. 6 A shows a group of archegonia in an apical depression. Mucilage mounds are seen around the younger ones, while in the older archegonia they have disappeared (cf. also Text-fig. 4 D-F). They represent a localized development of the mucilage covering the apical region, which becomes raised and finally perforated (Pl. VI o; Text-fig. 6 B). The mucilage mounds are apparent long before the cruciate cover-cells separate (Text-fig. 6 A, a) and are not a product of the contents of the archegonial neck. In hand-sections of fresh material stained in methylene blue they appear funnel-shaped (Text-fig. 6 c, showing the extrusion of the neck canal cells), the sloping surface layers absorbing the stain very deeply while the central region seems to have a more watery consistency. The funnel tends to shrink during fixation, but its features are sometimes clearly seen even in microtome sections (cf. Pl. VI o).

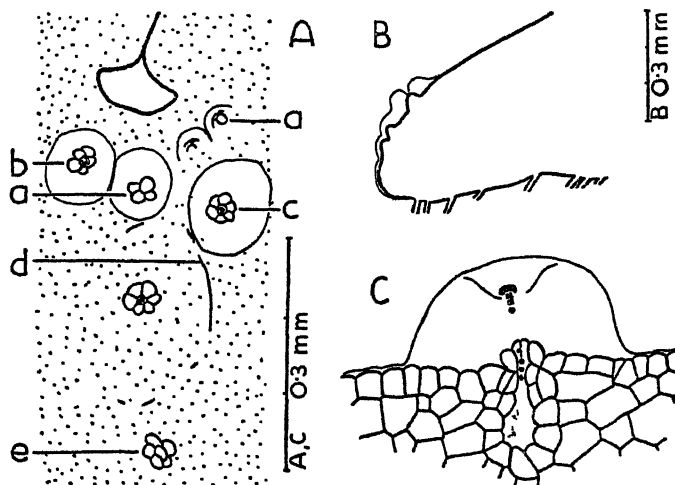
An abnormal double archegonium with two rows of central cells within a common wall was observed (Pl. VI p, centre right). Pande (1934) has described a similar case in *Notothylas levieri*.

(d) *Time of appearance of reproductive organs*. The dates given below are based on observations made on cultures and on material in nature. The development of sex-organs commences in September with the appearance of antheridia; the earliest archegonia have been observed in October. No further archegonia are developed in a lobe after an embryo has become established.

Production of either type of sex-organ usually ceases in May, although occasional antheridia may be seen as late as July. The earliest embryos observed were found in November; the earliest record of dehiscence was obtained in March. The main period of spore-production is from June to September, although occasional sporophytes may survive till December.

### IX. The eruption of the sporophyte

During the investigation of the dehiscence mechanism it was observed that the majority of sporophytes bear a cap of dead gametophyte tissue at their apices. A raised 'calyptra' is mentioned only for *Eubrya* in the current

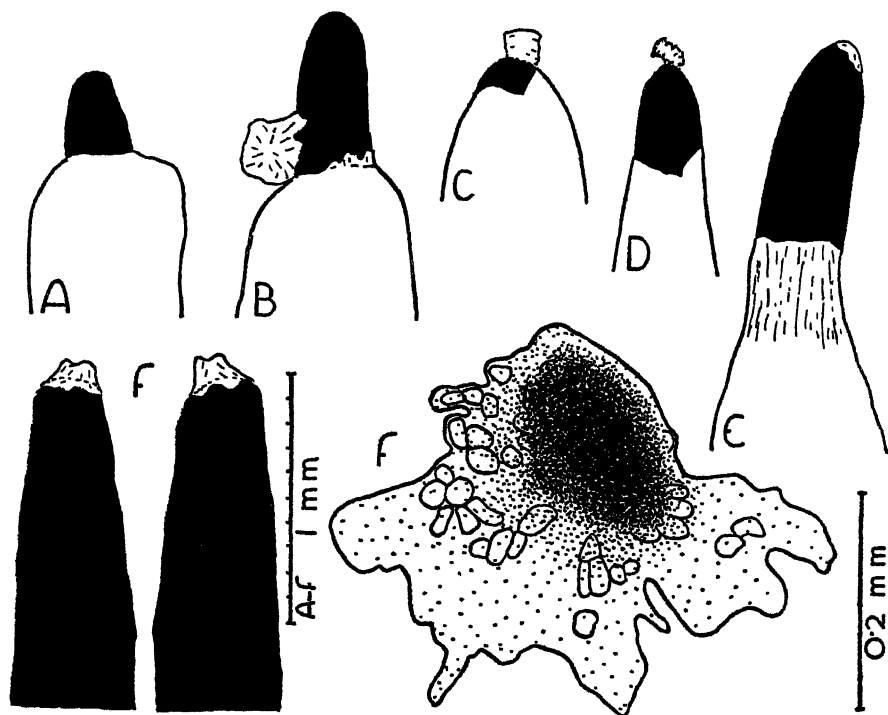


TEXT-FIG. 6. *A. laevis*. A. Apical depression with archegonia. *a*, cover-cells still in position; *b*, opening archegonium; *c*, open archegonium; *d*, remains of mucilage mound; *e*, old archegonium. B. Thallus apex with three archegonia, showing mucilage. C. Hand-section of fresh archegonium, showing extrusion of neck canal cells and mucilage mound.

literature, but the presence of such a structure in *Anthoceros* appears to have been generally recognized during the last century; Nees v. Esenbeck (1838) and Hofmeister (1851, with figures) both mention it.

The fleshy sheath which overarches the developing embryo is in part formed by tissue of the completely embedded archegonium, but mainly by surrounding thallus-cells. Some authors have, therefore, preferred to speak of it as an 'involucre' or 'perichaetium', although in the strict sense these latter terms are also inapplicable. Sometimes the extruding sporophyte tip simply punctures this sheath (Text-fig. 7 A), but usually the top is pushed aside (B) or detached as a slimy plug (C). This may be left behind (B), but it mostly remains permanently on the tip of the sporophyte (D-F), soon drying and becoming brown in colour, while the tissue breaks down. Text-fig. 7 F shows the cap of F more highly magnified. Some cell-walls are still visible, but the bulk consists of mucilaginous material harbouring abundant bacteria and fungi, if conditions are not too dry.

The appearance of the torn upper end of the sheath varies. The aperture may be stretched (A) or split (C, D) to let the wider subapical portions of the capsule through. Frequently the sheath dries at the top and appears as a brownish membrane (E) closely applied to the sporophyte. The shape of the mouth of the 'involucre' has been used as a diagnostic feature to distinguish species of *Anthoceros* (cf., for example, Cesares-Gil (1919) on



TEXT-FIG. 7. *A. laevis*. Diagram illustrating diverse methods of sporophyte eruption and 'calyptra' structure.

*A. beltrami*), but the degree of variation observed in *A. laevis* points to its unsuitability.

Once the sheath is open, the capsule continues to push up through it until basal growth ceases. If the mouth fits tightly around the capsule complications may arise. In one instance half of the sheath had been carried up as a ring around the subapical portion of the capsule. Not infrequently, if the capsule remains confined at the mouth of the sheath, the younger portions become folded up inside and ultimately burst out laterally (cf. Text-fig. 9 D, of *A. punctatus*). Such sporophytes are permanently distorted. This appears to have happened to the enlarged sporophyte of *A. fusiformis* pictured by Campbell (1924). Sometimes a whole series of zigzag folds may be seen.

Occasionally the sheaths of two or very rarely of three adjacent capsules

coalesce. This, the 'geminate' condition of certain authors, presumably results from the development of embryos from several archegonia of the same complex.

### Discussion

The investigated material agrees with Linné's description of *Anthoceros laevis* and the observable characteristics of the type-specimen.

The nomenclature of the growth-forms described in § II has to be considered. The original description (Watson, 1920) and Dr. Watson's exsiccata of the forma *aquatica* (Watson) Macvicar (1926) indicate that this represents a slightly etiolated plant from very moist habitats. If the type of nomenclature proposed by Buch (cf. Richards, 1945) were applied to the variation found in this species it would afford names such as *A. laevis* forma *aquatica-etiolata-ventro-tuberosa* for the plant illustrated in Text-fig. 1 B. This does not appear desirable, and since microclimatic factors tend to mass most of the growth-forms together in an individual locality no special nomenclature for them is here attempted.

The Dawlish plant, at the most, differs from the others in showing a greater tendency to produce a particular type of tuber. Since this is neither restricted to the Dawlish material nor constantly present in it, confusion would arise if the plant were given the rank of a variety.

*A. laevis* appears to have been much confused with *A. dichotomus*. As here understood (§ III) the latter is a very distinctive plant even in exsiccata. Most of the material ascribed to it, like that from Dawlish, seems to belong to *A. laevis*. Exsiccata labelled *A. dichotomus* by various collectors from such southern European localities as Madeira, Hérault, and Firenze cannot be distinguished from *A. laevis*; all of them, for instance, have small papillate spores. Cesares-Gil (1919) divides *A. dichotomus* into a f. *lata* and a f. *stricta*, but he considers it difficult or even impossible to distinguish the former from *A. laevis*.

In 1937 Schiffner issued material of a new species *A. incrassatus* (V. Schiffner, Hep. eur. exsicc. 1087, Prov. Como, Oct. 30, 1901). It has some broad subterminal tubers and possesses the spores of *A. laevis*, to which it belongs. Müller (1940) referred it to *A. dichotomus*.

There can be no doubt as to the dioecism (heterothallism) of the material investigated here. Many of the descriptions referring to *A. laevis* as monoecious are probably based on an analogy with *A. punctatus*, others, like Tindall's observations on the Dawlish plants, are no doubt due to confusion of young antheridial cavities with female organs. There remain the diagram of Cesares-Gil and the data given by such excellent observers as Nees v. Esenbeck (1838), and the existence of a monoecious, and possibly annual (cf. Nicholson, 1911), form of this species cannot be completely gainsaid. It might have arisen by polyploidy. The only evidence for possible polyploidy is Lörbeer's (1924) chromosome count and Hofmeister's (1851) observation of spore diads amongst tetrads.

The biological significance of the method of sperm discharge is no doubt the same as in *Pellia epiphylla* (Walton, 1943). The function, if any, of the archegonial mucilage funnel is obscure. It finds an analogy in the mucilage envelope and funnel around the mature macrogametophytes of Marsilia. In the frequent presence of a raised calyptra *Anthoceros* is intermediate between Hepaticae and Musci.

### Summary

A summary of this work, based on a critical investigation of *Anthoceros laevis*, may be given in the form of a modified diagnosis:

Perennial. Not found on basic soils. Thalli without mucilage cavities, ordinarily closely attached to the soil by rhizoids, their lobes becoming concave by mutual pressure, ascending when crowded; under moist conditions attachment is slight and branching very irregular; sometimes with narrow upright etiolated lobes. Lateral, subterminal, or ventral tubers frequent.

Diocious. Chromosome complement  $2n = 5$  (4 autosomes + 1 small sex-chromosome). Fertile male lobes narrower than female. Sex-organs in rows behind the apices. Antheridia usually 2–4 per cavity, their walls composed of numerous small cells, not arranged in tiers.

Dehiscing sporophytes from 0.7 to 9 cm. (usually 3–4 cm.) long, breaking open by one or two slits not reaching the apex, valves twisting spirally (cf. Pl. VI A).<sup>1</sup> Spores yellow, with short papillae on all faces, largest on the free face; max. diameter 30–47  $\mu$  (usually about 40  $\mu$ ). Spores produced from March to December (usually June–September).

Certain aspects of the morphology of the sex-organs and the presence of a calyptra are also described.

## 2. THE BRITISH BLACK-SPORED SPECIES AND THE GENUS ANTHOCEROS

### Introduction

Apart from *Anthoceros punctatus* L. four other species belonging to the black-spored section of the genus (viz. *A. husnoti* Steph., *A. stableri* Steph., *A. crispulus* (Mont.) Duin, and *A. longicapsulis* Steph.) have been established during the last 60 years and claimed to occur in Britain. *A. longicapsulis* has never received recognition, while doubts as to some of the others have been expressed by various authors (cf. especially Müller, 1912–16, 1940; Meylan, 1924; and Macvicar, 1926), Nicholson (1911) going so far as to say that it is doubtful whether modern authors have left anything which can be properly referred to *A. punctatus*. Müller maintains *A. punctatus*, *A. crispulus*, and *A. husnoti*; in addition to these Macvicar places *A. stableri* as a variety of *A. punctatus*.

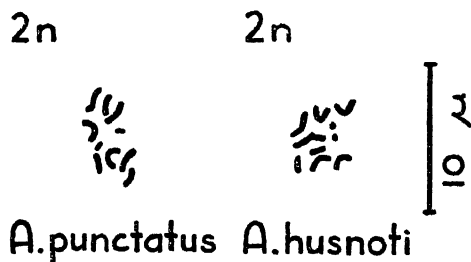
A critical investigation of living material was undertaken, and, where possible, original exsiccata were examined. It is concluded that there are only two British species of this section, viz. *A. punctatus* and *A. husnoti*. The

<sup>1</sup> The dehiscence will be described in detail in a further paper.

establishment by Stephani (1916) of the dubious genus *Aspiromitus* raises the question of the subdivision of the genus *Anthoceros*.

### I. Common features

The investigated forms agree in a number of respects besides those characteristic of the genus as a whole. The thicker parts of the thalli contain numerous cavities filled with mucilage. Externally the lobes can be distinguished at a glance from those of *A. laevis* in its typical condition by the



TEXT-FIG. 8. Squashes of diploid chromosome sets from meristematic regions of sporophytes.

presence of highly lacinate margins (cf. Pl. VI Q). Under certain conditions lamellate outgrowths may arise from their upper surface.

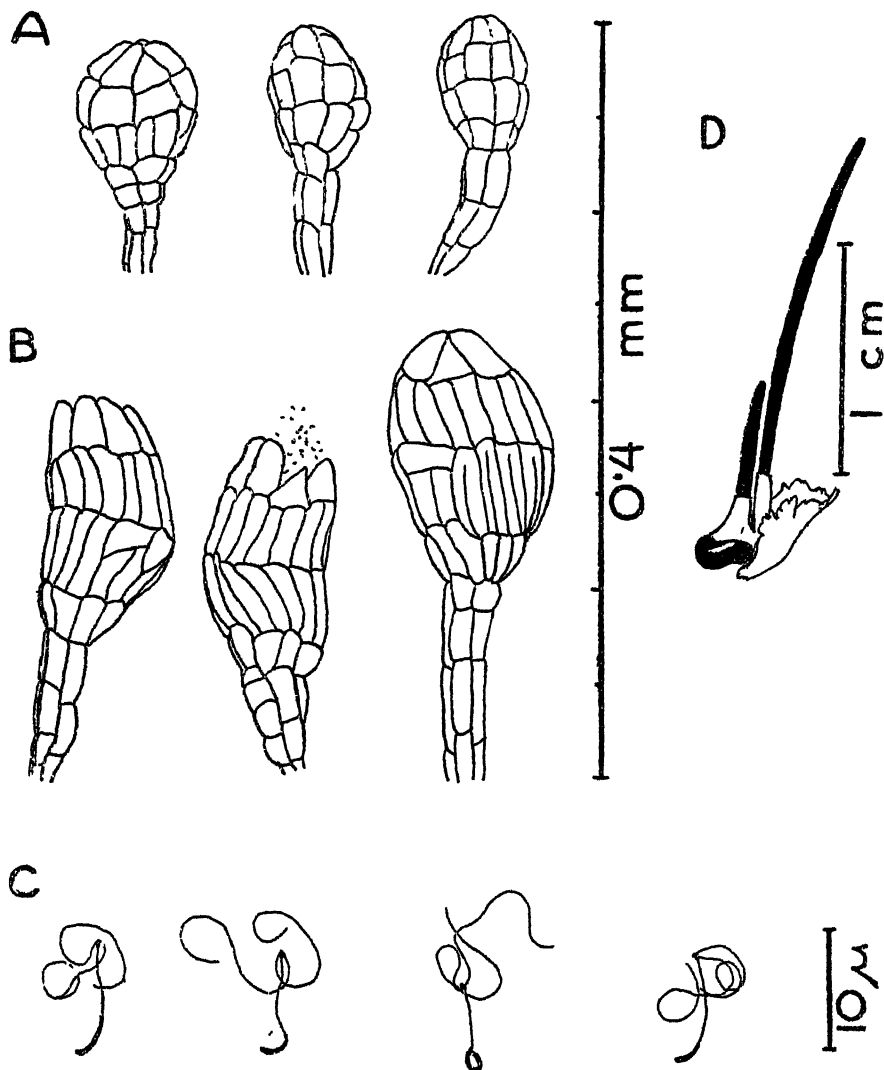
The plants are monoecious. The chromosome-sets in the diploid cells are composed of four pairs of large and one pair of identical small chromosomes (Text-fig. 8). Since the small chromosomes of *A. laevis* (cf. part 1) are differentiated into sex-chromosomes, it is reasonable to assume that here, too, they bear some, at least, of the genes concerned with the development of sex-organs. The presence of these smaller chromosomes may indicate that the monoecious condition has been derived from a dioecious one.

The arrangement of antheridial cavities and archegonia is in principle the same as described for *A. laevis* (Part 1, § IX). They arise in rows behind the growing-points. Development of male organs usually precedes that of the archegonia. The same growing-point later on suddenly switches over to their production, but the cause of the change is not known. It does not necessarily take place at the same time even in adjacent growing-points, so that male and female organs of the same age may sometimes be found lying side by side.

The antheridia have stalks composed of four rows of cells. Even at maturity the original horizontal divisions in the body are still noticeable, for the wall is formed of four tiers of cells (Text-fig. 9 A, B). By contrast the wall of the antheridium of *A. laevis* consists of numerous small irregularly arranged cells.

The antheridia open terminally by a separation of the cells of the uppermost tier (Text-fig. 9 B). The discharge and spreading of the spermatocytes

has been properly observed only in *A. husnoti* where it takes place exactly as described for *A. laevis*. The sperms of *A. husnoti* are like those of *A. laevis*, except that the (?) blepharoplast swelling is situated at the point of insertion



TEXT-FIG. 9. Mature antheridia of *A. punctatus* (A) and *A. husnoti* (B). C, sperms of *A. husnoti*; D, lobe of *A. punctatus* with two sporophytes, one of which has become folded inside its sheath and burst it laterally.

of the flagella (cf. Text-fig. 9 c with Text-fig. 5 c). The free sperms of *A. punctatus* have not been observed, but judging by the spermatocytes they are of the same size as those of *A. husnoti* and *A. laevis*.

Mucilage mounds occur around the archegonia as in *A. laevis*, although

they are often not very striking in *A. punctatus*. During the eruption of the sporophyte a 'calyptra' is almost invariably carried up. As in *A. laevis* (Part I, § IX) the capsule is sometimes caught at the mouth of its sheath and becomes folded inside, ultimately breaking out laterally (Text-fig. 9 D). Occasionally the sheaths of two or rarely three neighbouring sporophytes coalesce. At maturity the epidermis and contents of the capsule assume a dark brown-black colour. The dehiscence of the capsule will be described in a further paper.

Bower (1935, p. 19) remarks that the Anthocerotales appear to be singularly free from fungal infection. In this connexion it should be noted that the presence within the cells of protoplasts and thick-walled spores of what is presumably a chytrid is a very general feature of *A. punctatus* and sometimes also of *A. husnoti*. In the latter a filamentous fungal endophyte has several times been observed.

## II. *Anthoceros punctatus* L.

*Anthoceros punctatus* was described by Linné (1753) as the first species of *Anthoceros*. It is here, therefore, considered as the type of the genus. His description is: 'ANTHOCEROS frondibus indivisis sinuatis punctatis.' It is based on Dillen (1740) and Micheli's (1729) *A. minor*. England is given as one of the localities (cf. Dillen). The only labelled specimen in Linné's herbarium (bottom of sheet 1272.1) was probably not received until 1767 from Schreber (cf. Part I, § I).

The thalli are again very variable. Small plants, and especially those growing under relatively dry conditions in fields, seem covered with large numbers of crowded, minute, upright growths. This appearance is due to various features, viz. (a) the numerous crowded lobes of the thallus turned up at their tips and margins; (b) the numerous lacinate marginal outgrowths; (c) the presence of outgrowths from the upper surface in the form of minute lobes or frequently ridges of cells running longitudinally on the thallus-lobes (cf. also the key in Gottsche (1858): 'Superficie frondis in lobulos adventivos excrescente—*A. punctatus*'); (d) the roofs of the mucilage and antheridial cavities may break down in the older parts of the thalli, giving rise to depressions (cf. Müller, 1940); (e) the openings of the antheridial chambers often have a short neck; (f) the presence of developing embryos. It is to plants of this type that the synonym *A. crispulus* (Mont.) Duin applies; this has been checked with specimens in Schiffner, Hep. eur. exsicc. (*legit* Ch. et R. Duin).

Young plants collected at loc. 8 were planted on (a) the original New Red Sandstone soil, (b) garden soil (Aug. 9, 1946) and cultured under otherwise identical conditions. Six weeks later (Sept. 28, 1946) the biggest plant in culture (a) had a diameter of 0.8 cm., the typical *A. 'crispulus'* appearance, and the bulk of the sporophytes had turned black and partially dehiscent, the biggest reaching a length of 1 cm. (usually 5–6 mm.). In culture (b) the biggest plant had a diameter of 1.8 cm., with more spreading lobes with proportionally fewer lamellae from the upper surface. Most sporophytes

were still green, although some that were up to 2 cm. long had started to dehisce. The plants in this culture had thus assumed the characteristics assigned to *A. punctatus* in floras.

If grown under very moist conditions (for example, on agar, cf. Pl. VI Q, top) the plants appear hemispherical when viewed from the side owing to the upward growth of the central crowded lobes. The latter are exceedingly delicate, the marginal portions being only one cell thick. The fine outgrowths from the borders give these lobes a feathery appearance. Outgrowths from the upper surface are uncommon. The superficial tissue is composed of very elongate cells, separating easily from each other.

The production of sporophytes is very prolific in *A. punctatus*. In smaller plants the entire thallus-surface frequently appears studded with capsules, the development of which completely exhausts the gametophyte. Rink (1935) has shown that the growth of *Anthoceros* sporophytes is not impeded if photosynthesis is prevented. Similar experiments of my own on *A. laevis* corroborate this, except that no ripe spores have been obtained from capsules grown under such circumstances. These results are not incompatible with but are supplementary to those of Campbell (1917). Together with the observations on *A. punctatus* they demonstrate the extensive parasitism of the sporophytes on the gametophytes. In *A. punctatus* as a rule, both in agar and soil cultures, the gametophytes die as the sporophytes reach maturity, i.e. the plants are monocarpic. They are, therefore, obligate annuals when growing in the field, the normal growth period being probably June–January. The length of the sporophyte is here usually proportional to the size of the thallus and this is related to the fact that they cease growth as the latter becomes exhausted. They survive the gametophyte for a short period only.

The spores are brown-black in colour, the surface being reticulate on all faces, while numerous forked spines arise from the ridges of the reticulum on the free face. The max. diameter is 40–60 $\mu$ , usually *c.* 50 $\mu$ .

Linné's (1753) third species, *A. multifidus*, is based on Dillen (1740) only. The liverwort figured there (labelled *A. multifidus* in Linné's own copy) is clearly *Riccardia multifida*, thus *A. multifidus* is a synonym of the former. The names *A. punctatus*  $\beta$  *multifidus* (Nees v. Esenbeck, 1838) and *A. multifidus* (in a recent Russian work) have been used, probably in different senses, to denote *A. punctatus* pro parte. They must be rejected.

### III. *Anthoceros husnoti* Steph.

That *A. husnoti* is distinct from *A. punctatus* can easily be demonstrated by growing the two plants side by side on an agar-medium (Pl. VI Q). The plants of *A. husnoti* are far larger and thicker than those of the other species. *A. husnoti* normally grows in rather moister habitats than those frequented by *A. punctatus*, often together with *A. laevis*. Young crowded plants growing under such conditions are aptly described by Macvicar (1926) as 'broadly goblet shaped, ascending to suberect from a depressed centre, divided to the middle into . . . lobes . . .'. In the driest habitats frequented by

the species the lobes are fleshy, horizontal, and more firmly attached to the substratum. As in *A. punctatus* surface outgrowths, mainly in the form of longitudinal ridges but also lobes, are then fairly abundant. Surface regeneration, apart from that taking place from the margin, ensues on lobes partially killed by transitory unfavourable conditions. Plants showing such regeneration are frequently very reminiscent of *A. 'crispulus'*.

The form growing under very moist conditions (cf. Pl. VI Q, bottom) is irregularly branched with a practically smooth surface. The 'moist etiolated' form has narrow upright lobes with small pinnately arranged laterals.

Up to 22 antheridia have been counted in an antheridial cavity. It is difficult to give a minimum number because additional antheridia are invariably produced as proliferations from the bases of the stalks of the older ones, but there are probably never less than 8. The antheridial cavities in this species and in *A. punctatus* (with 4–14 antheridia per cavity) can be likened to 'mixed' fern sori, whereas *A. laevis* would illustrate the 'simple' condition. The antheridia themselves afford an excellent means of distinction between *A. husnoti* and *A. punctatus* (Text-fig. 9 A, B). In the former the body, when mature, is 110–150 $\mu$  (usually c. 120 $\mu$ ), in the latter 50–90 $\mu$  (usually c. 80 $\mu$ ) long.

The size of the sporophytes is exceedingly variable. The thalli are not exhausted by their formation, so that *A. husnoti* is perennial. The largest sporophyte was found in September 1944 growing in locality 2; it measured 14 cm. in length, 4.5 cm. of the basal region being still green. It is thus comparable with the largest specimens of *A. fusiformis* (Campbell, 1924). The smallest dehiscing sporophyte seen was only 0.5 cm. long; 4–6 cm. can be considered as the most usual length.

Dehiscing sporophytes of a wide range of size may frequently be found even on one and the same thallus. This, in conjunction with the size difference between *A. husnoti* and *A. punctatus*, suggested that the former might be a polyploid form of the latter, and that sporophytes with different chromosome numbers might occur together on the same thallus. The chromosome complement is, however, numerically identical in the two species (cf. Text-fig. 8) and no aberrations were observed. The spores are in all respects like those of *A. punctatus*.

Since *A. husnoti* is perennial and it is impossible to define the limits of an individual plant, measurements of the diameter of plants as given for *A. punctatus* are useless.

The species was established by Stephani in 1888 (Rev. bryol., xv. 49). *A. stableri* Steph. dates from 1895. The most characteristic feature of this species is given as the presence of 12–20 antheridia in the cavities. Müller (1912–16) pointed out that this was a feature of *A. husnoti*. Macvicar (1926), misquoting Müller, refers to it as *A. punctatus* forma *stableri* (Steph.) Macv. Material from S. Westmoreland (legit G. Stabler, Oct. 19, 1881) has the antheridia and other thallus characteristics of *A. husnoti*, of which *A. stableri* is thus a synonym. The principal feature of *A. longicapsulis* Steph. (1916)

is that the capsules are 6 cm. long. The original description states 'antheridia destructa'. It is clearly a synonym of *A. husnoti*. The latter species has thus been described on three different occasions by the same author, firstly, for the most part on the features of the thallus, secondly, on those of the antheridia, and thirdly, on those of the sporophytes. Moreover, Stephani made it the type of a new genus (see below).

*A. husnoti* is very similar to but normally 'greater in all its parts' than *A. punctatus*. Both species are highly plastic. A small specimen of *A. husnoti* might be mistaken for *A. punctatus*. A glance at the antheridia, some of which are usually to be found, will, however, quickly decide the species. It is a matter of individual opinion whether *A. husnoti* is considered a separate species, or, as Meylan (1924) suggests, merely a large variety of *A. punctatus*.

#### IV. The genus *Anthoceros* L.

If reliable descriptions of species of *Anthoceros* (excl. *Megaceros* Campb.) are examined, they are seen to fall into two sections: (a) a series in which the capsules, spores, and pseudo-elaters are brown-black at maturity and mucilage cavities are present in the thalli, and (b) a series in which the capsules are brown and the spores and pseudo-elaters yellow at maturity, while the thalli lack mucilage cavities (cf. also Bartlett, 1928). It is reasonable to regard the Linnean species *A. punctatus* and *A. laevis* as the respective types of these two sections.

Stephani (1916) established the genus *Aspiromitus* and gave as the first and only European species *A. husnoti*, which is thus presumably the type. It is interesting to note that its synonyms *A. stableri* Steph. and *A. longicapsulis* Steph., as well as *A. punctatus*, are left in the genus *Anthoceros*. It has never been made clear (cf. Goebel, 1928) how *Aspiromitus* is to be distinguished from *Anthoceros*. Rink (1935), following Goebel, considers the antheridia, in which the cells of the wall are arranged in tiers, to afford the best criterion. On the strength of the single species studied by him, he gives as an additional feature the presence of a neck at the aperture of the antheridial chambers. Such a neck is sometimes present and sometimes absent in all the British species of *Anthoceros* and is, therefore, of no systematic importance. The antheridial characteristics are identical with those here demonstrated for *A. punctatus* (Text-fig. 9 A), the type of the genus *Anthoceros*. As pointed out above, the type of the genus *Aspiromitus*, *A. husnoti*, could easily be considered to be merely a variety of *A. punctatus*. Stephani's genus *Aspiromitus* is thus completely untenable and must be rejected. The species referred to it should be included with *Anthoceros*; all those of which a reliable description is available (incl. the dioecious *Anthoceros sampalocensis* (Burgeff) n. comb.) belong to the black-spored section.

A thorough investigation of the many species of *Anthoceros* is necessary before subgenera can be firmly established. Besides the features afforded by the colour and mucilage cavities, the members of the black-spored section may all prove to be characterized by the possession of antheridia in which

the cells of the wall are in distinct tiers and by the regular arrangement of the cells in the foot of the sporophyte (cf. Bartlett, 1928). This last feature has been found to hold for the British species of this group, as compared with the irregular arrangement of the cells in the sporophyte foot of *A. laevis*. The species with black spores might be included in a subgenus *Euanthoceros*, so named because the type of the subgenus, *A. punctatus* L., is also the type of the genus.

The members of the yellow-spored section (type *A. laevis* L.) might ultimately and advantageously receive recognition as a separate genus. A name such as *Phaeoceros* might be suitable and in keeping with the names of the other genera of *Anthocerotaceae* emend. Müller (1940).

### Summary

There are only two native black-spored species of *Anthoceros*. The investigations made on them are summarized in the following modified diagnoses:

1. *A. punctatus* L. (syn. *A. crispulus* (Mont.) Duin). Monocarpic annual, growth period June–January. Not on basic soils. Thalli with mucilage cavities, diameter up to 2 cm., lobes up to 20 (usually 8–12), cells thick in the middle, margins lacinate, with surface outgrowths especially in plants from relatively dry situations.

Monoecious. Chromosome complement  $1n = 5$  (four larger and one small chromosome). Sex organs produced in rows behind the apices, archegonia in front of antheridial cavities, the latter with 4–14 antheridia, body of antheridium  $50\text{--}90\mu$  (usually *c.*  $80\mu$ ) long, with the cells of the wall in four tiers.

Dehiscing sporophytes 0.4–3 cm. (usually 1–2 cm.) long, calyptra usually present, dehiscence by one or two slits not reaching the apex or by partial decay, valves twisting spirally.<sup>1</sup> Spores brown-black, reticulate on all faces, with furcate spines arising from the ridges of the reticulum on the free face; max. diameter  $40\text{--}60\mu$  (usually *c.*  $50\mu$ ).

2. *A. husnoti* Steph. (syn. *A. stableri* Steph., *A. longicapsulis* Steph.). Differs from *A. punctatus* in the following points:

Perennial. Thalli larger, lobes up to 30 (usually 12–22), cells thick in the middle. Antheridial cavities with 8–22 antheridia, their bodies  $110\text{--}150\mu$  (usually *c.*  $120\mu$ ) long. Dehiscing sporophytes 0.5–14 cm. (usually 4–6 cm.) long. Spores produced from April to September, occasionally as late as December.

The genus *Aspiromitus* Steph. is rejected, its species being referred to *Anthoceros*. The establishment of subgenera is discussed.

<sup>1</sup> See footnote on p. 256.

## APPENDIX

*Key to the British species of Anthoceros*

1. Thalli without mucilage-cavities. Spores yellow *A. laevis*  
     Thalli with mucilage-cavities. Spores brown-black 2
2. Lobes up to 20 (usually 8–12) cells thick in middle, body of  
     antheridia 50–90  $\mu$  (80  $\mu$ ) long, capsules 0.4–3 cm. (1–2 cm.) long *A. punctatus*  
     Lobes up to 30 (usually 12–22) cells thick in middle, body of  
     antheridia 110–150  $\mu$  (120  $\mu$ ) long, capsules 0.5–1.4 cm. (4–6 cm.)  
     long *A. lusnoti*

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## EXPLANATION OF PLATE VI

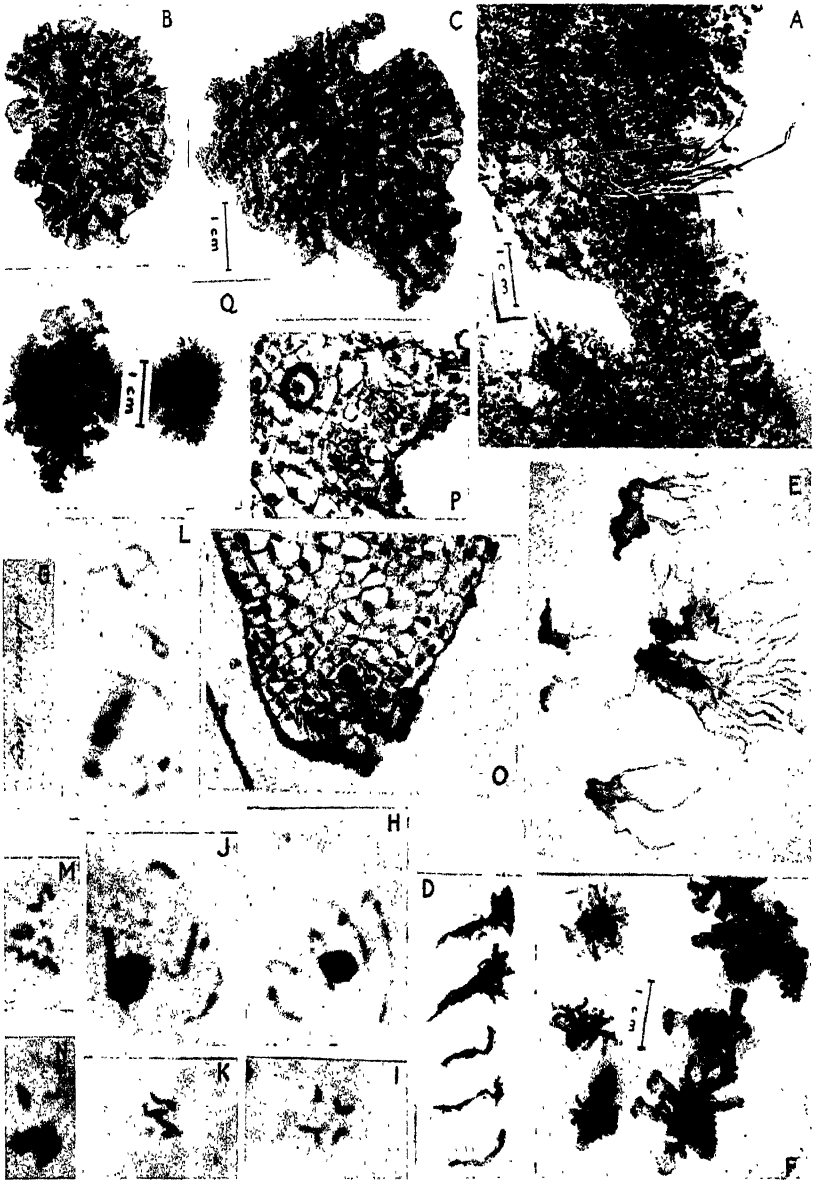
Illustrating J. Proskauer's paper on *Anthoceros*

A-P, *Anthoceros laevis*. A, Material from Aberystwyth grown on Old Red Sandstone soil from Dawlish. Narrow-lobed plants bearing antheridia in the lower left-hand corner. Capsules showing dehiscence by one or two slits. July 1947. B, Typical rosette of thallus-lobes (with archegonia, loc. 5). C, Material growing on vertical cliff at Dawlish. D, 'Clawed' lobes (culture, loc. 6). E, Extremely etiolated plants grown under moist conditions. The two plants at the bottom bear antheridia (culture, loc. 1). F, Regenerates of thallus-lobes planted on nutrient agar on Oct. 24, 1946, photographed on Dec. 12, 1946. The lower three plants bear antheridia, the remainder archegonia. (A-F,  $\times 0.9$ .) G, Label in Linné's hand on the typesheet in his herbarium. ( $\times 0.6$ .) H-N, Photomicrographs of squash preparations of chromosomes. (Zeiss Fluorit 100 N.A. 1.3, K. 10) ( $\times 1,800$ .) H, I; J, K; L, M, Pro- and metaphases of male, female, and diploid sets respectively. N, First meiotic metaphase. O, Vertical section through a growing-point and a closed archegonium with mucilage mound. (Fixed: formalin acetic-alcohol, embedded: tertiary butyl alcohol method, stained: safranin, Bismarck brown, fast green.) P, Horizontal section through two normal and one double archegonium. The two younger archegonia are cut through the region of the neck, neck canal cells still in position. O, P, Photomicrographs. ( $\times 175$ .)

Q, *Anthoceros punctatus* (top) and *Anthoceros husnoti* (bottom) grown side by side on nutrient agar. ( $\times 0.9$ .)



[TOP



[BOTTOM

PROSKAUER—ANTHOCEROS I



# Studies in the Genus *Fucus*

## I. On the Structure and Chemical Composition of *Fucus vesiculosus* from Three Scottish Localities

BY

BETTY L. MOSS

(*Westfield College*)

With three Figures in the Text

### INTRODUCTION

STRUCTURAL differentiation accompanies development of a thallus of *Fucus vesiculosus*. Products of its metabolism have been estimated by previous workers,<sup>1</sup> without relation to the developmental stage or structural features of the plants analysed, or to the habitats from which they were obtained. A previous study of the anatomical structure of *Fucus vesiculosus* and *Fucus serratus* from widely separated localities around the shores of Great Britain<sup>2</sup> had shown that secondary differentiation of the thallus appeared to be most marked in plants from exposed habitats.

For the present investigation, plants were collected off the west coast of Argyllshire, at Loch Melfort, Aird's Point, and Cullipool. These three habitats were selected by the Scottish Seaweed Research Association on account of their varying degrees of exposure to wave action, although they experience similar weather and tidal phenomena and have a similar substrate. Typical mature plants of *Fucus vesiculosus* were gathered from each locality during September 1946, and although the exact age of the thalli is unknown, they were apparently of several seasons growth. In September the fruiting period was nearing completion, and the plants selected for this study had lost their recently borne receptacles.

From Loch Melfort plants were gathered at Kames Bay, a *sheltered shore* where thalli are subjected to little movement other than a gradual rise and fall with the tide. In this habitat *Fucus vesiculosus* was intermingled with a zone of *Ascophyllum nodosum*, the latter species predominating. Both species were attached to small granite boulders eroded from the mainland. At the time of collection it was observed that complete attaching discs were easily removed from the substrate. (They are usually so firmly attached that pulling the stipe removes part of the substrate together with the attaching disc.) Subsequent examination of the attaching discs revealed the presence of rudiments of numerous adventitious branches arising from the under surfaces which had been adjacent to the substrate, an unusual phenomenon probably related to the sheltered habitat.

<sup>1</sup> Kylin 1913, Butler 1931, Haas and Hill 1933.

<sup>2</sup> In preparation for publication.

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Aird's Point is situated approximately 30 miles up the Firth of Lorne. It receives *moderate wave action*, for the islands of Lismore and Mull afford protection from the Atlantic Ocean. *Fucus vesiculosus*, attached to granite promontories of the mainland, formed a well-defined zone between *Ascophyllum nodosum* and *Fucus serratus*.

At Cullipool *Fucus vesiculosus* was also attached to granite promontories of the mainland. These were exposed to *very strong wave action*, for the shore borders the Atlantic Ocean. In this habitat a narrow zone of *Fucus vesiculosus* occurred between *Fucus spiralis* and *Fucus serratus*.

The aims of the present investigation have been:

- (a) To study the anatomy of mature plants of *Fucus vesiculosus* from the three habitats differing in their degrees of exposure to wave action.
- (b) To investigate the chemical composition of different regions of thalli from each locality. Presumably previous workers have analysed complete plants, thus ignoring differences in anatomical structure which occur throughout an entire thallus.
- (c) To determine the chemical composition of similar regions of thalli from localities differing in exposure to wave action.

Material for anatomical study was fixed in formalin-acetic-alcohol. Air-dried material was also soaked in distilled water and hardened in 50 per cent. alcohol before sectioning. Preparation of material for chemical analyses is outlined later.

#### EXTERNAL FEATURES

The main external features of a typical plant from each locality are summarized in Table I. From the table it is apparent that a thallus from Loch Melfort is  $1\frac{1}{2}$  times the length of a plant from Cullipool, and yet possesses only  $\frac{1}{3}$  the total number of dichotomies. Thus, while the length of a thallus decreases from sheltered loch to open sea, the total number of dichotomies increases. Yet the number of dichotomies in vertical succession from the disc to the farthest apex is the same (12) in plants from each locality. Hence it follows that branching of the laterals is greater in plants from the open sea, and at the same time each interdichotomy is shorter.

TABLE I  
*Comparison of the External Features of Fucus vesiculosus from Three Scottish Localities*

Habitat.	Length of thallus (dried and pressed).	Total no. dichots.	No. dichots in vertical succession.	Length of thallus from which wings eroded.	Maximum width of wings.	Vesicles.
Loch Melfort (sheltered)	74 cm.	152	12	44 cm.	1.0 cm.	Small; few
Aird's Point (moderately exposed)	58 cm.	412	12	27 cm.	1.5 cm.	Small; several pairs per interdichotomy
Cullipool (exposed)	60 cm.	450	12	15 cm.	2.5 cm.	Large; several pairs per interdichotomy

The wings become eroded from the midrib in the lower part of a mature thallus. It is remarkable that the greatest erosion should be found in plants from the *sheltered* habitat (Table I). Wings and bladders are worn away throughout more than half the length of thalli from Loch Melfort. Approximately half the length of thalli from Aird's Point is devoid of wings and bladders, whereas thalli from Cullipool have lost both bladders and wings for only a quarter of their entire length. Plants from Cullipool have very persistent bladders, for the wings may be partially removed up to half the length of the thallus, although complete vesicles are still attached to the midrib.

Thalli from the sheltered Loch are narrow and have small vesicles, usually one or two pairs of the latter occurring between each dichotomy. Plants from Cullipool have several pairs of large vesicles between each dichotomy (about 1.5 cm. in length as compared with a length of 0.8 cm. in thalli from Loch Melfort). The width of wings at their widest point in thalli from the open sea is also approximately twice that of plants from the sheltered loch (Table I). Thus from sheltered loch to open sea there is an increase in the width of the thallus and also in the size and number of vesicles.

Although differences in the external form of thalli have been outlined, Fig. 1, showing the tips of thalli from each of the three selected localities, is included to demonstrate that plants from these localities are of essentially the same growth form of *Fucus vesiculosus* (apparently var. *divaricatus* Goodenough and Woodward).

#### ANATOMICAL STRUCTURE

A Fucoid thallus has apical growth, and in a groove at the tip of each branch is a large meristematic cell. From the products of its segmentation three primary tissues are differentiated. These are known respectively as meristoderm, cortex, and medulla. There is a gradation of heavily pigmented cortical cells into the central medulla, and the intervening region will henceforward be referred to as the 'transition zone'. It is in this transition zone that hyphae first originate. They arise as small outgrowths from primary cells and hence are secondary in origin. These outgrowths increase in length and undergo septation. Eventually the hyphae form long filaments of cells, twining like rope-fibres, amongst the medullary cells and cells of the transition zone. Hyphae increase in number towards the attaching disc, largely as a result of the juxtaposition of hyphae from above. Filaments of medullary cells separate close behind the apex, and towards the attaching disc their longitudinal walls thicken considerably.

During development of the thallus the cortex increases through the activity of the meristoderm. But in the plants of *Fucus vesiculosus* from these three habitats, secondary cortex arises chiefly as the result of both radial and tangential divisions in the primary cortical cells. Tangential divisions predominate until a compact tissue of regular radial rows of secondary cortical cells is formed. The lower regions of the thallus become surrounded

by secondary cortex, enclosing primary cells of the medulla entwined by numerous hyphae (secondary in origin).

The distance behind the apices at which secondary differentiation commences varies in plants collected from these three localities. In plants from

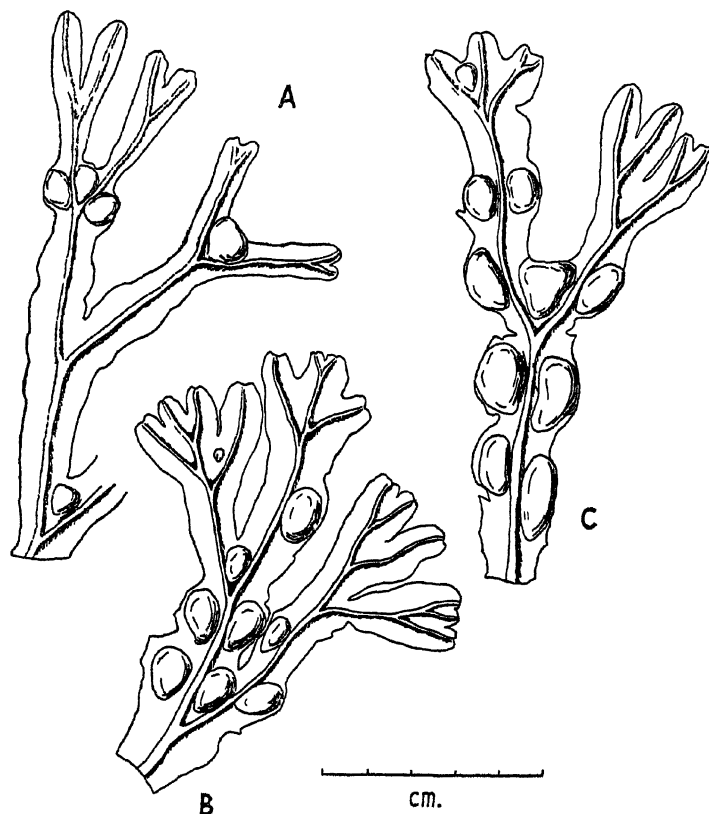


FIG. 1. Tips of plants of *Fucus vesiculosus* from three Scottish localities.

- A. From Loch Melfort (sheltered).
- B. From Aird's Point (medium exposure).
- C. From Cullipool (exposed).

Aird's Point and Cullipool hyphae can be found as close to the apex as 0.4 cm. Hyphae do not appear in the transition zone of plants from the sheltered loch until approximately 1 cm. behind the apex. It is remarkable that primary cortex should persist so long in the latter plants. It has been found up to 35 cm. below the apex, into the lower part of the thalli from which the wings are eroded (Table I). By contrast, in plants from the open sea numerous divisions in the primary cortical cells can be seen as close to the apex as 8 cm. It is these plants which have the widest wings and the

largest and most numerous vesicles, all bounded by secondary cortex. Plants from the open sea thus exhibit the maximum development of secondary cortex.

Secondary cortex in stipes from Loch Melfort, i.e. sections taken half-way between the attaching disc and the first dichotomy above it, often shows irregular development and forms approximately one-tenth the radius of the stipe. It may be 10 cells wide, whereas secondary cortex in stipes from Aird's Point reaches 16 cells, and from Cullipool as much as 25 cells in width (radially). In stipes from both Aird's Point and Cullipool secondary cortex constitutes approximately one-fifth the radius, i.e. twice as much as found in stipes from Loch Melfort. A comparison between the central tissues of stipes from the three localities is shown in Table II. The size and number of medullary cells (i.e. primary cells) per unit area is similar for all stipes, but in plants from the open sea there are approximately twice as many hyphae (i.e. secondary cells) scattered amongst the medullary cells.

TABLE II  
*Comparison of the Central Tissues of Stipes of Fucus vesiculosus  
from Three Scottish Localities*

Habitat.	Av. no. hyphae per unit area.	Av. no. medullary cells per unit area.	Diameter of medullary cells in units.
Loch Melfort	102	8	18
Aird's Point	116	9	18
Cullipool	182	9	20

The differences established in plants of comparable development passing from sheltered loch to open sea can be summarized as follows:

- (1) Reduction in total length.
- (2) Greater development of lateral branching.
- (3) Larger and more numerous vesicles.
- (4) Less erosion of wings and laterals.
- (5) Earlier development of hyphae at the apex.
- (6) Increase in the development of secondary thickening and differentiation of the thallus.

#### CHEMICAL COMPOSITION

##### *Method*

Approximately 50 lb. of mature plants of *Fucus vesiculosus* were collected from each locality during the first two weeks of September 1946. Thalli were cut off just above the attaching discs, so that these organs are not included in the samples. As already stated, plants were gathered at the end of the fruiting season, but any remaining fertile tips were discarded. Each thallus, from each locality, was divided into three parts:

- (i) *Distal*, i.e. the apical 5–6 cm. of each branch; a region consisting chiefly of primary tissues.

- (ii) *Proximal*, i.e. the lower part of the thalli from which both wings and bladders were eroded. (As previously noted in Table I, the length of this region varies, being over half the total length of thalli from Loch Melfort, compared with one-quarter the total length of Cullipool.) This sample contains secondary cortex enclosing numerous hyphae intertwining around primary medullary cells of the midrib.
- (iii) *Middle*, i.e. the whole of the thalli between (i) and (ii). This sample contains both wings and midrib. Secondary tissues increase towards its lower limits.

Thus a total of nine samples were collected from the three habitats. Dry-weight determinations were carried out at the Oban outstation of the Scottish Seaweed Research Association and here the samples were dried. Later, they were ground to a fine powder in a Christy and Norris mill fitted with a  $\frac{1}{64}$ -in. perforated plate screen. The analytical methods used are those developed by the Scottish Seaweed Research Association,<sup>1</sup> to whom I am indebted for help with the chemical analyses.

Results of the analyses are set out in Tables III–VI. In Table IV the results are expressed as a percentage of the dry weight and are of technical interest where quantities of dried weed are to be used for extraction. In Tables V–VI the results are expressed as a percentage of the fresh weight. This is the essential basis for biological comparison of the plants here analysed.

### *Dry weight*

For dry-weight determinations, approximately 100 g. of fresh weed from each sample were dried at 100° C. to constant weight.

From Table III it is apparent that the dry weight increases from distal to proximal regions of plants from the same locality, i.e. increase in dry weight accompanies secondary differentiation of the thallus. Thalli from each locality give similar percentage dry weights for distal regions, but the dry

TABLE III  
*Percentage of the Dry Weights of Fucus vesiculosus*  
(expressed as per cent. fresh weight)

Habitat.	Region.	Dry weight.
Loch Melfort {	Distal	23·6
	Middle	24·2
	Proximal	25·6
Aird's Point {	Distal	25·1
	Middle	27·8
	Proximal	31·4
Cullipool {	Distal	24·1
	Middle	33·3
	Proximal	33·0

Not yet published.

weights of both middle and proximal samples increase from sheltered loch to open sea. The greatest difference between the dry weights of distal and proximal samples from the same locality is shown in plants from the open sea (cf. 2 per cent. Loch Melfort, 7 per cent. Aird's Point, and 10 per cent. Cullipool). Plants in which secondary differentiation is most marked exhibit the greatest difference between the percentage dry weights of distal and proximal regions of thalli.

It is interesting to note that middle samples from Loch Melfort and Aird's Point give values intermediate between distal and proximal samples from the same locality. In contrast, the middle sample from Cullipool is never the proximal sample from the same habitat. It is in plants from this locality that the development of both hyphae and secondary cortex sets in closest to the apex.

TABLE IV  
*Chemical Composition of Fucus vesiculosus expressed as Per Cent.  
Dry Weight*

Habitat.	Region.	Ash.	Total organic nitrogen.	Mannitol.	Lami- narin.	Alginic acid.	Total.
Loch Melfort	Distal	17.2	0.7	15.7	9.0	16.6	59.2
	Middle	18.3	0.82	12.5	5.0	12.0	48.6
	Proximal	15.8	1.07	8.8	2.8	10.8	39.3
Aird's Point	Distal	16.1	0.84	13.2	7.1	17.7	54.9
	Middle	16.4	0.93	13.6	5.7	10.0	46.6
	Proximal	14.4	1.33	9.8	3.3	9.4	38.2
Cullipool	Distal	20.5	1.04	13.8	5.1	20.7	61.1
	Middle	17.1	0.95	13.8	5.8	12.0	49.6
	Proximal	16.0	1.1	10.1	5.0	8.0	39.2

#### *Total ash*

The total ash content of the three samples from Loch Melfort is very similar. Middle samples from Aird's Point and Cullipool show a slightly higher ash content than either distal or proximal samples. Similar regions of thalli from the different localities show an increase in ash content from sheltered loch to open sea (Table VI).

#### *Organic nitrogen*

As can be seen from Table V, the organic nitrogen content increases from distal to proximal regions of thalli from each locality. The increase is most marked in plants from the sheltered loch, where there is more than twice as much organic nitrogen in the proximal as in the distal sample. Whether this increase in organic nitrogen from the young tips to the stipe is connected with any form of protein storage in the latter region of thalli is unknown. The organic nitrogen content of both distal and middle samples increases from sheltered loch to open sea, while the proximal samples from each locality give similar results.

*Mannitol*

The percentage mannitol decreases from distal to proximal regions of thalli from Loch Melfort (Table V). Cells of both primary and secondary cortex are rich in pigments and probably constitute the main photosynthetic tissues of the thallus. As the number of hyphae increases from the tip of the thallus to the attaching disc, and primary cortex persists in Loch Melfort plants throughout both distal and middle regions, it follows that the proportion of

TABLE V

*Comparison between Three Regions of Thalli from the Same Locality expressed as Per Cent. Fresh Weight*

Habitat.	Region.	Ash.	Total organic nitrogen.	Mannitol.	Laminarin.	Alginic acid.
Loch Melfort	Distal	4.0	0.17	3.7	2.1	3.9
	Middle	4.4	0.2	3.0	1.2	2.9
	Proximal	4.0	0.27	2.3	0.7	2.7
Aird's Point	Distal	4.0	0.2	3.3	1.8	4.4
	Middle	4.6	0.26	3.8	1.6	2.8
	Proximal	4.5	0.42	3.0	1.0	2.9
Cullipool	Distal	4.9	0.25	3.3	1.2	5.0
	Middle	5.7	0.32	4.6	1.9	4.0
	Proximal	5.3	0.36	3.3	1.6	2.6

photosynthetic tissue decreases from the tip of the thallus. This decrease in the proportion of photosynthetic tissue accompanies the decrease in mannitol content observed in Loch Melfort samples. Loss of the wings means loss of photosynthetic tissue, for cortex forms more than half the diameter of the wings. This loss of wings, together with the poor development of secondary cortex, is correlated with the low percentage of mannitol obtained in the proximal sample from Loch Melfort.

Plants from both Aird's Point and Cullipool give the highest percentage mannitol in the middle samples (Table V). It is in the middle samples of thalli from these habitats that the wings reach their maximum width, and where the bladders are both large and numerous. Both increase the surface area of the thallus, and so increase the proportion of cortex available for photosynthesis.

Associated with the increase in the development of secondary cortex in thalli from sheltered loch to open sea, there is a progressive increase in mannitol content both of middle and proximal samples. The proximal sample from Aird's Point more nearly approximates that from Cullipool. In stipes from both these habitats secondary cortex constitutes two-fifths the diameter, whereas in thalli from Loch Melfort only one-fifth the diameter of the stipes consists of secondary cortex. This decrease in cortical development is associated with a lower mannitol content (Table VI).

*Laminarin*

Laminarin content shows many similarities to mannitol content. Both substances decrease from distal to proximal regions of thalli from Loch Melfort (Table V). The distal sample from this habitat has a higher percentage of laminarin than any other sample analysed; it has three times the laminarin content of the proximal sample from the same locality. Although distal regions of Loch Melfort plants are richest in laminarin, proximal regions of the same plants give the lowest laminarin content.

From sheltered loch to open sea, the distal samples show a decrease in laminarin, as in mannitol content (Table VI). In a sheltered loch the water is still and clear so that the tips of thalli receive more light than in water

TABLE VI

*Comparison between Similar Regions of Thalli from Different Habitats expressed as Per Cent. Fresh Weight*

Region.	Habitat.	Ash.	Total organic nitrogen.	Mannitol.	Laminarin.	Alginic acid.
Distal	Loch Melfort	4.0	0.17	3.7	2.1	3.9
	Aird's Point	4.0	0.2	3.3	1.8	4.4
	Cullipool	4.9	0.25	3.3	1.2	5.0
Middle	Loch Melfort	4.4	0.2	3.0	1.2	2.9
	Aird's Point	4.6	0.26	3.8	1.6	2.8
	Cullipool	5.7	0.32	4.6	1.9	4.0
Proximal	Loch Melfort	4.0	0.27	2.3	0.7	2.7
	Aird's Point	4.5	0.42	3.0	1.0	2.9
	Cullipool	5.3	0.36	3.3	1.6	2.6

churned by wave action. As a result, tips of thalli from Loch Melfort may have a higher rate of carbon assimilation accounting for the high value of laminarin obtained. On the other hand, thalli from the open sea exhibit more branching, and secondary differentiation sets in closer to the apex. Hence substances such as laminarin and mannitol may be utilized in processes of growth and new cell formation and so do not accumulate to give high percentages in the distal samples.

Just as plants from the open sea have a maximum mannitol content in the middle samples of thalli, so is laminarin here at its maximum (cf. 1.2, 1.9, and 1.6 per cent. for distal, middle, and proximal samples respectively from Cullipool, Table V). Also in both middle and proximal samples there is an increase in laminarin content from sheltered loch to open sea. Similarly, as outlined for mannitol, the percentage laminarin can be correlated with the development of photosynthetic tissue.

*Alginic acid*

Alginic acid content decreases from distal to proximal regions of thalli from all localities as shown in Tables IV and V. For example, the distal regions

of thalli from Cullipool have 20.7 per cent. alginic acid compared with 8.0 per cent. in proximal regions of the same plants expressed on an anhydrous basis, or 5.0 per cent. compared with 2.6 per cent. calculated on a fresh-weight basis. Thus the percentage alginic acid is highest at the tips of thalli in regions of primary tissues which are exposed to violent slashings by wave

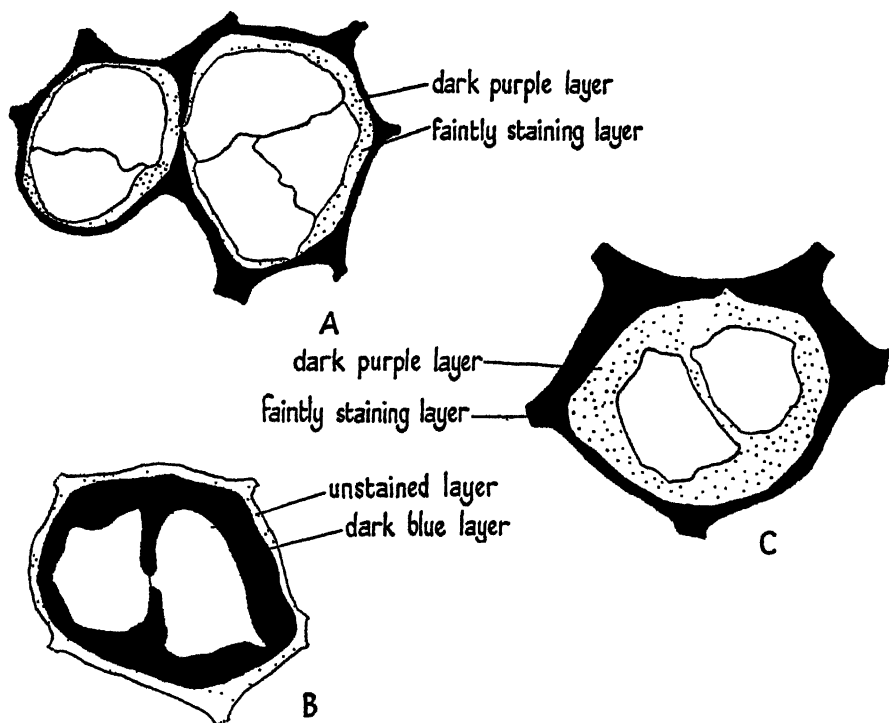


FIG. 2

FIG. 2a. Reaction of the cortical cell walls to gentian violet before alginic acid extraction.

FIG. 2b. Reaction of the cortical cell walls to iodine and concentrated sulphuric acid before alginic acid extraction.

FIG. 2c. Reaction of the cortical cell walls to gentian violet after alginic acid extraction.

action and yet remain undamaged. Both distal and middle samples of thalli show progressive increase in alginic acid content from sheltered loch to open sea (Table VI).

Alginic acid is generally regarded as a cell-wall constituent. When Stanford first extracted algin from *Laminaria* he obtained 'a gelatinous mass, consisting of a thick, glutinous, gummy liquid, containing the cellular fabric of the plant completely broken up'. He goes on to discuss the difficulty of filtration and says that '... cells to be removed are so minute ...'. He was thus aware that the residue contained cell walls. An examination of the residue obtained after prolonged alginic acid extraction from these samples of finely powdered *Fucus vesiculosus* revealed the presence of both hyphal and cortical cells.

Their walls showed no visible loss of material. Therefore, sections of air-dried *Fucus vesiculosus* were cut and treated as for alginic acid extraction. The reactions of the cell walls to gentian violet, and to iodine and concentrated sulphuric acid, were observed both before and after extraction.

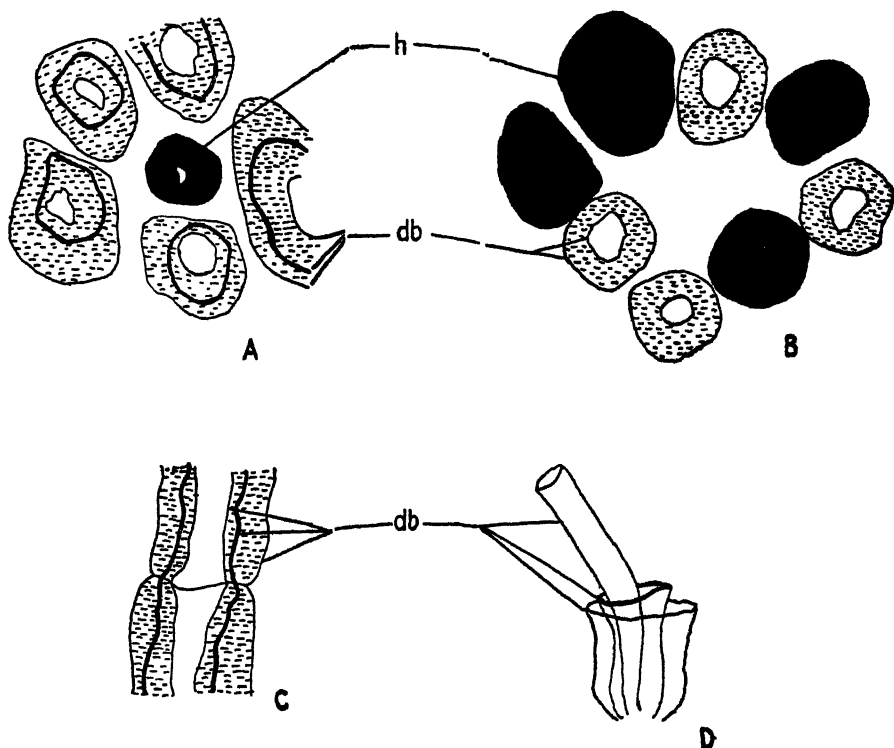


FIG. 3. Action of iodine and concentrated sulphuric acid upon the central cells of stipe.

- A. Medullary cells and hyphae in T.S. before alginic acid extraction.
- B. Medullary cells and hyphae in T.S. after alginic acid extraction.
- C. Medullary cell in L.S. before alginic acid extraction.
- D. Medullary cell in L.S. after alginic acid extraction.

*h* = hyphal walls stained dark blue.

*db* = layers of medullary cell walls stained dark blue.

Fig. 2a shows the effect of gentian violet upon the cell walls of the cortex before alginic acid extraction. The middle lamella stains deep purple, while the layer adjacent to the protoplast is only faintly stained. The latter gives a blue reaction with iodine and concentrated sulphuric acid (Fig. 2b), a reaction said to indicate the presence of cellulose in higher plants. After soaking in N/5 sulphuric acid the cortical walls become very swollen but give the same staining reactions. The same two layers can be distinguished in the walls of cortical cells after alginic acid extraction (Fig. 2c). Thus alginic acid does not appear to be a major constituent of the cell walls of the cortex of *Fucus vesiculosus*.

Fig. 3 A and C show the effect of iodine and concentrated sulphuric acid upon the central tissues of the stipe before alginic acid extraction. The hyphal walls give a distinct cellulose reaction, and concentric blue staining layers are obtained in the walls of medullary cells.<sup>1</sup> After alginic acid extraction, the central tissues of the stipe fall apart, for there has been removal of the intercellular matrix, together with partial (Fig. 3 B) or complete (Fig. 3 D) removal of the layers separating what appear to be cellulose membranes of the medullary cells. This method suggests that alginic acid does not form the major part of the skeletal framework of *Fucus vesiculosus*.

Both hyphae and cortex increase from the apex of thalli towards the attaching disc. The apparent absence of alginic acid from the cell walls of these tissues would account for the lower values obtained in proximal samples. Instead, in the lower regions of the thallus, where severe pulling strains are experienced, the cell walls consist of cellulose-like material.

#### *Unestimated residue*

The analytical methods employed account for approximately 58 per cent. of the dry matter of distal samples, 47 per cent. of middle samples, and only 39 per cent. of proximal samples. The unestimated residue increases from distal to proximal regions of thalli from each locality, i.e. with secondary differentiation of the thallus. Removal of nitrogen, mannitol, laminarin, and alginic acid leaves a residue in which cell walls of hyphae, cortex, and those layers of the medullary cell walls which give a blue reaction with iodine and concentrated sulphuric acid can be discerned. It is hoped that in the future the chemical nature of these cell walls may be elucidated and methods for their accurate estimation be obtained.

#### SUMMARY

It has been shown that plants of *Fucus vesiculosus* collected from three Scottish localities with different degrees of exposure to wave action exhibit variations in both external features and anatomical structure. Secondary differentiation of the thallus is most marked in plants from the open sea.

As anatomical differentiation proceeds from the apex to the base of a thallus, so does the chemical composition vary; the percentage dry weight and organic nitrogen (expressed as percentage fresh weight) increase while alginic acid decreases.

The chemical composition of similar regions of thalli from the three selected localities exhibit differences, some of which are correlated with variations in anatomical development. Passing from sheltered loch to open sea there is an increase in the percentage dry weight, organic nitrogen, mannitol, and laminarin of middle and proximal samples (expressed as percentage fresh weight). The distal and middle samples show an increase in alginic acid content from sheltered loch to open sea.

The plants for this investigation were selected from three habitats differing

<sup>1</sup> As shown by Naylor and Russell-Wells (1934).

in exposure to wave action. Yet accompanying differences in wave action there are doubtless variations in temperature and supply of nutriments which presumably will influence development of thalli. The results obtained may be due to the integration of all these factors.

This investigation was carried out in the Department of Botany, Westfield College, during the tenure of a College Research Studentship. It was suggested to me by Dr. E. M. Delf, to whom I wish to express my sincere thanks. I also wish to thank the Scottish Seaweed Research Association for covering the cost of collecting the material used in this investigation, and for assistance with the chemical analyses. The latter formed part of the programme of research on seaweed undertaken by the Scottish Seaweed Research Association. I am indebted to the Association for permission to publish.

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# A Physiological Study of Leaf Growth

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## INTRODUCTION

THE yield of a green plant, expressed in its most general form as dry-matter accumulation, depends on the efficiency of utilization of solar energy by the plant and on the size of the assimilating system. The efficiency of the assimilating system is appropriately measured by the net assimilation rate and its size by the leaf area, the expression of the summation of the actual leaf area over the period of growth. Studies of field crops (Watson, D. J., 1947) have shown that yield is determined mainly by leaf area and that net assimilation rate plays a minor part in causing variations in yield. Since leaf growth is the immediate determinant of leaf area it becomes of interest to investigate the physiology of this process. The present investigation is an attempt to explore some of the factors concerned in leaf growth.

The process of leaf initiation and development is in itself a complex one and comprises at least three definite stages. The first stage is the formation by the apical meristem of the leaf initial, a small group of cells constituting a local meristem of limited activity. The second stage is one of active cell

division by which the initial gives rise to the cells and tissues of the leaf. As cell division comes to an end it is followed by the third stage, when cell extension causes a considerable increase in the size of the leaf, accompanied by the appearance of intercellular spaces and the final differentiation of the individual cells. The three stages are not necessarily as sharply separated in the development of an individual leaf as this schematic description might suggest. Each stage makes a definite contribution to leaf area, however, and whilst closely linked to the others each is possibly dependent on a distinct complex of factors. An investigation of leaf growth must clearly take into account these stages of development and in particular must distinguish between the effects of cell division and cell extension in determining the final area of a leaf.

A study of the literature shows relatively few investigations of leaf growth as such, and a complete review will not be attempted. It will suffice to indicate briefly the main factors which may be concerned. These include light, temperature, water-supply, mineral nutrients (including trace elements), supply of carbohydrate and other metabolites to the growing leaf (including changes in nutrient balance between different parts of the plant), and possibly the supply of growth-regulating substances.

Temperature in the sub-optimal range has been shown to have a direct effect on meristematic activity as expressed in the rate of production of leaf initials (see Gregory, 1928; Ashby and Oxley, 1935; Watson and Baptiste, 1938). At higher, supra-optimal temperatures the rate falls off, probably owing to inhibition of cell division. The relation between temperature and rate of formation of leaf initials appears to be similar to that found for other processes of growth. The effect of temperature on the further development of the initials (cell division and extension) seems to have been little studied, but Ashby and Oxley (*loc. cit.*) found that frond area in *Lemna* is independent of temperature over a wide range.

Apart from its photosynthetic effect light is a determining factor in the development of the leaf from the leaf initial. Priestley (1925) found that in total darkness the expansion of leaf initials is almost completely inhibited in most plants, whilst brief exposures to light (insufficient to cause photosynthesis or even chlorophyll production) result in leaf expansion. He concluded that light has a photochemical action on some metabolite present in the leaves, converting it into a substance essential for growth and cell division. Evidence for the existence of a photochemically produced leaf-growth substance has also been obtained by Gregory (1921, 1928) and by Went (1938). This formative effect of light on leaf growth takes place at very low light intensity and is probably not of practical, although of considerable theoretical, interest. At higher intensities light has complex effects on leaf structure. Studies of sun and shade structure have not always clearly differentiated between the effects of light and of varying water-supply to the leaves. There is no doubt, however, that light has a definite formative influence apart from any associated differences in water-supply. The effect of different light intensities on the cell size and the total number of cells of the mature leaf

appears to be considerable (Watson, R. W., 1942), although the relations have not been systematically elucidated. There are also differences in cell and tissue differentiation which are bound up with light intensity. The possibility that light may also have a direct effect on the formation of leaf initials is indicated by the experiments of Ashby and Oxley (1935) and White (1936), who found that light at low intensities increased the relative multiplication rate of *Lemna* with no further increase at light intensities above about 750 foot-candles. Working with *Sporodela* (a similar waterplant) Gorham (1945) found that light increased the relative multiplication rate even when the plant was provided with sucrose.

Whilst leaf growth clearly depends on the supply of water, mineral nutrients, carbohydrates, and other metabolites, there is little direct information since most experimental work has been concerned with the relation between these factors and the more general aspects of growth. The relation between leaf growth and specific growth substances remains obscure. Reference has already been made to the evidence for a photochemically produced substance essential for the normal development of leaf initials. According to Avery (1935) auxin only controls the growth of midrib and veins in leaves and does not affect development of the mesophyll. Various substances reported to have effects on plant growth, including heteroauxin, have been shown to have no significant effect on the growth of isolated leaves of rye in sterile culture (de Ropp, 1945).

In the present work it was decided to confine attention to the effect of water-supply, nitrogen, and salt (sodium chloride) on leaf growth in sugar-beet grown in pots. Salt was included because of the marked response of sugar-beet to manurial applications of this compound. Sugar-beet was selected as experimental material since it is an important agricultural crop, the physiology of which has been much studied, and is convenient in producing from a single meristem a regular succession of leaves of simple shape relatively easy to measure. Supplementary observations were made on leaf material from a number of field crops receiving various manurial treatments, in order to provide a wider background to the results of the pot experiment with sugar-beet. Some preliminary experiments on the relation between light and leaf growth were also carried out and will be briefly reported. In order to distinguish between effects on cell division and cell extension during growth a simple microscopic method was developed to estimate the total number of cells in a leaf.

Additional results on more general aspects of growth were derived from the main experiments, and these will also be reported since they are related to the problem of leaf growth.

#### EXPERIMENTS WITH SUGAR-BEET IN POT CULTURE

##### *Aim and design of experiments*

The object of the experiments was to examine the effect on leaf growth of two levels of water-supply combined with two levels of nitrogen and two

levels of salt (sodium chloride). Two varieties of sugar-beet were used, grown from two batches of seed from single plants selected from homogeneous races kindly supplied by G. D. H. Bell, Plant Breeding Institute, Cambridge. The variety Hilleshög was grown in all combinations of the factors under investigation. For comparison the variety Kleinwanzleben was employed, which differs from Hilleshög in the larger average size of its leaves. In order to keep the size of the experiment within practical limits Kleinwanzleben was grown at the higher level of nitrogen and salt, and only the effect of high and low water régime was investigated.

The experiment consisted of six blocks, of which three blocks were harvested in July, about half-way through the growing period. At this point the water régime of half of the remaining plants was changed over so that plants formerly receiving high now received low water, and plants formerly receiving low now received high water. The other half of the plants continued to receive the same water régime as in the first period. The experiment was concluded in September when the remaining three blocks were harvested.

The experimental treatments are best represented by the combination of the following factors:

- $w_1$  = high water-supply in first period of growth;
- $w_2$  = high water-supply in second period of growth;
- $n$  = higher level of nitrogen;
- $s$  = higher level of salt.

The lower levels of nitrogen and salt (corresponding to the amounts originally present in the soil, without addition) are represented by the absence of  $n$  and  $s$ . Similarly low water-supply is represented by the absence of designation. When all factors are present at the lower level the treatment is conventionally represented as (1). Whilst the representation of the manurial treatments is obvious, that of the four water régimes should be noted.

- (1) = low water-supply over whole period
  - $w_1 w_2$  = high water-supply over whole period
  - $w_1$  = high water-supply in first period, then low
  - $w_2$  = low water-supply in first period, then high
- } constant water régime.  
} alternating water régime.

The plan of the experiment will be made clearer by the following summarized statement. It will be seen that there is threefold replication of the treatments.

*Sugar-beet: Hilleshög.* Sown May 2, 1946. All pots with basal P and K; 72 pots in all.

Treatments	3 blocks.	3 blocks.
	(1)	(1)
	$s$	$s$
	$n$	$n$
	$ns$	$ns$
	$w_1$	$w_1$
	$w_1 s$	$w_1 s$

3 blocks.	3 blocks.
$w_1 n$	$w_1 n$
$w_1 ns$	$w_1 ns$
	$w_2$
	$w_2 s$
	$w_2 n$
	$w_2 ns$
	$w_1 w_2$
	$w_1 w_2 s$
	$w_1 w_2 n$
	$w_1 w_2 ns$
Harvested	Harvested
July 17	Sept. 9

*Sugar-beet: Kleinwanzleben.* Sown May 2, 1946. All pots with basal NPK and salt; 18 pots in all.

3 blocks.	3 blocks.
(1)(ns)	(1)(ns)
$w_1(ns)$	$w_1(ns)$
	$w_2(ns)$
	$w_1 w_2(ns)$
Harvested	Harvested
July 17	Sept. 9

The primary data are too bulky for reproduction and records are therefore usually given in the form of means per plant for each treatment together with a statement of the mean treatment effects per plant (main effects and first-order interactions). The conventional representation of actual treatments by small letters and of treatment effects by capital letters has been adopted.

### *Treatments*

The plants were grown in large glazed pots with bottom drainage in Rothamsted soil (clay with flints) containing an admixture of 10 per cent. clean sand. Each pot contained 10.5 kg. of the air-dry soil-sand mixture initially. All manurial additions were made by mixing appropriate quantities of the required salts (in solution) with the weighed amount of dry soil before putting it into the pot. The total amount of solution added to each pot was 150 ml., and pots not receiving the full manurial treatment received the corresponding amount of distilled water.

*Nutrient supply.* All pots were given a basic manurial addition of P and K in the form of  $K_2HPO_4$  (1.25 g. equivalent to 0.51 g.  $P_2O_5$  and 0.67 g.  $K_2O$  per pot).

Plants at the lower level of nitrogen received no further addition. Those at the higher level received 1 g. N per pot given as  $(NH_4)_2SO_4$ .

Plants at the lower level of salt received no further addition. Those at the higher level received 2.5 g.  $Na_2O$  (4.7 g. NaCl) per pot.

### *Water régime*

It would be theoretically most desirable to compare leaf growth in plants grown at a constant high and a constant low level of soil moisture. There is, however, no practical method of maintaining, in pots, a constant level of

soil moisture other than field capacity. The following method was therefore adopted in order to create two levels of water-supply. All pots were just saturated with water at sowing by adding the required quantity of water as found by direct determination of the field capacity of the soil. Plants under high water régime were then fully watered in the normal manner, keeping the soil at the bottom of the pot moist to the touch but avoiding any through drainage. Water was entirely withheld from the low-water series until the plants showed signs of severe wilting. Each pot was then brought back to saturation by adding the required amount of water calculated on the assumption that in this particular soil approximately 50 per cent. of the moisture at saturation is available to the plant. The substantial correctness of this assumption was roughly confirmed by direct weighing of the pots showing severe wilting. All pots were weighed initially and a proportion was weighed at intervals in order to keep some check on the water balance, but the labour of weighing all pots regularly was too heavy to be undertaken, and in any case this method of checking is complicated by the increase in weight of the plant itself. In practice it was not difficult to bring pots back to field capacity when required by taking the moistness of the soil at the base of a pot as the criterion of saturation and exercising a little judgement in the amount of water to be added. A record was kept of the water added to each pot. The criterion of severe wilting in the low-water plants was the complete collapse of all but the three or four very youngest leaves before 10 a.m. Direct determination of water loss by weighing indicated that these symptoms occurred when the soil was very close to the permanent wilting-point. Sugar-beet is a plant which wilts rather readily but also readily recovers. Plants which show complete collapse in an afternoon frequently recover during the night, but the appearance of these symptoms in the early morning shows the approach of the danger-point. There was always complete recovery of the collapsed plants within 1-2 hours of watering.

#### *Cultural operations and watering*

The sugar-beet was sown on May 2, 6-8 'seeds' to a pot. The pots were placed in trucks which were at first kept in the greenhouse and later wheeled into the open air. The arrangement of blocks and of treatments within each block was randomized. By May 10 all pots showed germination, and after gradual hardening off between May 13 and May 20 the plants were moved outside permanently on May 20. The plants were thinned to 4 per pot on May 13, to 2 per pot on May 20, and finally to 1 per pot on May 29 to May 30. Three blocks were harvested on July 17 and the remainder on September 11. The plants were kept free from aphids by hand-cleansing and none showed signs of virus infection at the final harvest.

The trucks were provided with detachable transparent covers which could be put on at night or when there was rain during the day. The plants were thus entirely dependent on the measured amounts of distilled water which they received. The water régime is summarized in Table I. The mean

amount of water supplied to a plant of the continuous high-water series was 19.01 litres and to a plant of the continuous low-water series was 9.10 litres during the period May 2 to September 9. In general low-water plants received roughly half the amount of water supplied to corresponding treatments in the high-water series. This is also true of those plants in which there was alternation of high and low water régimes.

TABLE I  
*Amount of Water supplied to Sugar-beet (litres per plant)*

Variety.	Treatment.	May 2–Sept. 9 (whole period).	July 15–Sept. 9 (second period only).
Hilleshög	(1)	7.22	2.90
	s	6.79	2.81
	n	11.49	5.78
	ns	10.57	5.78
	w <sub>1</sub>	10.90	2.25
	w <sub>1</sub> s	11.20	2.54
	w <sub>1</sub> n	15.07	6.14
	w <sub>1</sub> ns	15.07	6.14
	w <sub>2</sub>	10.62	7.47
	w <sub>2</sub> s	10.46	7.38
	w <sub>2</sub> n	17.16	12.51
	w <sub>2</sub> ns	17.85	13.50
	w <sub>1</sub> w <sub>2</sub>	16.13	7.20
	w <sub>1</sub> w <sub>2</sub> s	16.22	7.22
	w <sub>1</sub> w <sub>2</sub> n	22.07	13.14
	w <sub>1</sub> w <sub>2</sub> ns	21.62	12.69
Kleinwanzleben	(1)(ns)	13.11	7.33
	w <sub>1</sub> (ns)	14.89	5.78
	w <sub>2</sub> (ns)	19.82	15.03
	w <sub>1</sub> w <sub>2</sub> (ns)	22.34	13.41

On the assumption that the amount of water supplied can be taken as a measure of water transpired, figures for water loss per sq. dm. of leaf surface have been calculated for the second growth period (July–September) (Table II).

TABLE II  
*Water-loss in Sugar-beet (Hilleshög) in period July–September*

Treatment.	Mean water loss (litres per sq. dm. leaf surface).
Continuous low water (1)	0.40
Continuous high water (w <sub>1</sub> w <sub>2</sub> )	0.80
Low water following high (w <sub>1</sub> )	0.38
High water following low (w <sub>2</sub> )	0.86
Low nitrogen (1)	0.80
High nitrogen (n)	0.56
No salt (1)	0.64
Salt (s)	0.61

Whilst the transpiration rate is higher when the water-supply is high and lower when the water-supply is restricted, there is no evidence that water régime has any permanent effect on transpiration rate. Nitrogen considerably

decreases transpiration rate, but salt is without effect. Field observations indicate that salt retards the onset of wilting in sugar-beet and there seemed to be some evidence of this effect in the present experiment. The records of wilting cover too short a period to provide definite confirmation of this.

### *Observations*

Throughout the experiment weekly records were made of the production of new leaves and the death of old leaves. New leaves were counted as soon as they were 1 in. in length; dead leaves were those which were completely yellow. The length and breadth of each 5th leaf was measured weekly in order to determine when the phase of rapid extension was over and to provide data for estimating the total leaf area. When rapid extension had ceased the final area of each 5th leaf was measured by direct pencil tracing whilst the leaf was attached to the plant. The area of the tracing was then determined by means of a planimeter. In some leaves a very slow expansion may continue almost until the leaf dies, so that 'final' area is a practical and not an absolute term. At the same time as the final area was measured a small sample of the leaf was taken by means of a cork-borer for estimating the number of cells in the leaf in the manner explained below.

### *Method of determining number of cells in the leaf*

The following method was used to estimate the approximate number of cells in the leaf. By means of a sharp cork-borer a sample of about 1 cm. diameter was taken from the lamina midway between the midrib and the margin at a point about one-third of the way up the leaf from the attachment of the petiole. The sample was placed at once in 70 per cent. alcohol and air removed by evacuation. The leaf material can be preserved in 70 per cent. alcohol until required.

To make a cell count the sample is mounted directly in saturated aqueous chloral hydrate with the upper (adaxial) surface of the leaf uppermost. The chloral hydrate clears the mount and renders the upper epidermal cells almost invisible. The uppermost palisade cells are very clearly visible under the microscope as regular almost isodiametric cells which are easy to count with the aid of a micrometer square in the eyepiece. The area of the micrometer square at the magnification employed must be determined by reference to a micrometer slide. The mean of four random fields is taken as the cell count in routine work. A single field should include not less than 10, nor more than 30-40, cells, and the magnification should be adjusted if necessary. Since the area of the leaf is known, the total number of upper palisade mesophyll cells is easily calculated from the cell count (number of cells per unit area). This is a measure of the total number of cells in the leaf, for in sugar-beet the cells of the leaf blade are laid down in a definite number of layers (nine in the material used in this experiment), which appears to be constant for a given variety. The arrangement of the mesophyll cells is very regular and there is roughly the same number of cells in each layer. Examina-

tion of material from a variety of treatments shows that the number of cell layers in the blade is quite constant even with very wide differences in the age, treatment, and total number of cells in the leaf.

The total number of upper palisade mesophyll cells, calculated from cell count and leaf area, is used throughout as the measure of the total number of cells per leaf. The real total number of cells is a multiple, of the order of 9-10, of this figure. Rough calculations indicate that allowance for the vascular bundles and the greater number of cell layers at the main veins would not seriously alter the total estimate.

Leaf material must be fully turgid when fixed in alcohol, otherwise the cells are difficult to count. There is a superficial contraction of 4 per cent. when the leaf is fixed and this is not altered by transfer to chloral hydrate. This contraction is allowed for when calculating the number of cells in the leaf.

## RESULTS

### *Formation of leaf initials*

The total number of leaves formed during the whole growing period (May-September) is recorded in Table III. The higher level of nitrogen markedly increases the activity of the apical meristem with consequent more rapid production of leaf initials. The rate of formation of new leaves

TABLE III  
*Production of Leaves in Sugar-beet (May 2-Sept. 9)*

	Treatment.	Mean number of leaves per plant.			
Hilleshög	(1)	32.0	Mean		34.29
	s	28.3	Main effects	$W_1$	-0.08
	n	37.0		$W_2$	+0.08
	ns	38.6		N	+10.66*
				S	-0.08
	$w_1$	30.6	Interactions	$W_1 W_2$	-0.58
	$w_1 s$	28.3		$W_1 N$	-1.66
	$w_1 n$	38.0		$W_2 N$	+1.83
	$w_1 ns$	41.0		$W_1 S$	+0.08
	$w_2$	24.0		$W_2 S$	+0.25
	$w_2 s$	28.3		NS	-0.50
	$w_2 n$	44.6		S.E.	1.13
	$w_2 ns$	41.6			
	$w_1 w_2$	28.3			
	$w_1 w_2 s$	31.6			
	$w_1 w_2 n$	40.0			
	$w_1 w_2 ns$	36.0			
Kleinwanzleben	(1)(ns)	17.3	Mean		15.58
	$w_1(ns)$	13.6	Main effects	$W_1$	-2.16
	$w_2(ns)$	16.0		$W_2$	+0.16
	$w_1 w_2(ns)$	15.3	Interactions	$W_1 W_2$	+1.50
				S.E.	1.26

appears, however, to be quite unaffected by water-supply or level of sodium chloride.

This picture is not altered when the two growing periods are considered separately as in Table IV. The preponderant effect in each period is that of nitrogen supply. During the first period low water-supply appears slightly to reduce the formation of new leaves in both varieties, but this probably represents merely a check in leaf expansion since the trend is reversed in the second period.

TABLE IV  
*Production of Leaves in Sugar-beet in Two Periods of Growth*

Treatment.		Mean number of leaves per plant.	
<i>First period: May 2–July 15</i>			
Hilleshög	(1)	18.1	Mean 20.67
	<i>s</i>	17.8	Main effects <i>W</i> +1.14*
	<i>n</i>	22.4	<i>N</i> +4.86*
	<i>ns</i>	22.1	<i>S</i> -0.36
	<i>w</i>	18.9	Interactions <i>WN</i> +0.52
	<i>ws</i>	18.2	<i>WS</i> 0.00
	<i>wn</i>	24.0	<i>NS</i> +0.14
	<i>wns</i>	23.9	S.E. 0.53
Kleinwanzleben	(1)( <i>ns</i> )	19.3	Mean 20.04
	<i>w</i> ( <i>ns</i> )	21.3	Main effects <i>W</i> +1.88
			S.E. 1.83
<i>Second period: July 15–September 9</i>			
Hilleshög	(1)	14.0	Mean 13.44
	<i>s</i>	9.3	Main effects <i>W</i> <sub>1</sub> -0.88
	<i>n</i>	16.0	<i>W</i> <sub>2</sub> -0.04
	<i>ns</i>	16.3	<i>N</i> +5.38*
			<i>S</i> +0.29
	<i>w</i> <sub>1</sub>	10.6	Interactions <i>W</i> <sub>1</sub> <i>W</i> <sub>2</sub> +0.04
	<i>w</i> <sub>1</sub> <i>s</i>	10.6	
	<i>w</i> <sub>1</sub> <i>n</i>	14.6	
	<i>w</i> <sub>1</sub> <i>ns</i>	16.0	
	<i>w</i> <sub>2</sub>	6.0	<i>W</i> <sub>1</sub> <i>N</i> -2.04*
	<i>w</i> <sub>2</sub> <i>s</i>	11.3	<i>W</i> <sub>2</sub> <i>N</i> +0.79
	<i>w</i> <sub>2</sub> <i>n</i>	19.6	<i>W</i> <sub>1</sub> <i>S</i> +0.38
	<i>w</i> <sub>2</sub> <i>ns</i>	18.3	<i>W</i> <sub>2</sub> <i>S</i> +1.04
			<i>NS</i> -0.38
			S.E. 0.76
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub>	11.0	
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> <i>s</i>	13.0	
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> <i>n</i>	14.3	
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> <i>ns</i>	13.6	
Kleinwanzleben	(1)( <i>ns</i> )	17.33	Mean 15.58
	<i>w</i> <sub>1</sub> ( <i>ns</i> )	13.66	Main effects <i>W</i> <sub>1</sub> -2.16
	<i>w</i> <sub>2</sub> ( <i>ns</i> )	16.00	<i>W</i> <sub>2</sub> +0.16
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> ( <i>ns</i> )	15.33	Interactions <i>W</i> <sub>1</sub> <i>W</i> <sub>2</sub> +1.50
			S.E. 1.26

The general effect of nitrogen is clearly seen in the alternating series, but the most interesting feature is the marked acceleration of leaf formation in the low-water/high-nitrogen plants when switched to a high water régime.

This is evidently connected with the relatively greater uptake of N by plants with low water-supply, and will be discussed in relation to the nitrogen figures in a later section.

A close relation between nitrogen supply and the rate of formation of leaf initials would be expected. That this aspect of meristematic activity should be relatively independent of water-supply is more surprising. It is obvious that the activity of the stem apex is ultimately related to, and affected by, water-supply, and the plants with alternating water régime provide evidence of this relation in certain conditions. In general, however, meristematic activity at the apex seems to be insensitive to water-level. This is probably mainly due to the ability of this region to withdraw water preferentially from other parts of the plant. This phenomenon is very obvious in sugar-beet; the youngest developing leaves close to the growing-point show no signs of wilting or flaccidity when the rest of the plant is completely collapsed from water loss. Probably other species of plants would show a closer dependence of meristematic activity on water-supply, for sugar-beet not only possesses a considerable internal supply of water in the tap-root but also shows drought resistance to a marked degree.

The mean length of life of the individual leaf is not significantly affected by the level of nitrogen or salt supply, or by the water régime except when there is alternation. The transition from high to low water régime slightly hastens the appearance of senescence (complete yellowing), whilst transition from low to high water régime delays the appearance of senescence. These effects, which are not very pronounced, are best shown by the mean number of dead leaves expressed as a percentage of the number of leaves produced in the whole growth period for the main treatments (Table V).

TABLE V

Treatment.	Number of dead leaves as % total number of leaves produced (Hilleshög).
Continuous low water (1)	22.0
Continuous high water ( $w_1 w_3$ )	21.5
High, followed by low water ( $w_1$ )	24.9
Low, followed by high water ( $w_3$ )	18.5
Low nitrogen (1)	21.6
High nitrogen ( $n$ )	21.9
Low salt (1)	22.2
High salt ( $s$ )	21.3
All treatments	21.7

### *Number of cells in leaf*

In Table VI the information concerning the total number of cells per leaf (in hundred thousands) is collected. As already noted, the figures should be multiplied by a factor of the order of 9–10 to give the correct magnitude. The figures for the 1st, 5th, and 10th leaves are the means of six replicates, those for the 15th and 20th leaves are the means of three replicates.

TABLE VI

*Mean Number of Cells per Leaf in Sugar-beet (in hundred thousands).  
Constant Water Régime*

	Treatment.	1st leaf.	5th leaf.	10th leaf.	15th leaf.	20th leaf.	Mean (all leaves).
Hilleshög	(1)	5.0	24.2	54.0	52.7	19.1	31.0
	<i>s</i>	5.1	24.9	48.4	55.1	18.4	30.4
	<i>n</i>	6.1	34.1	73.3	118.0	73.2	60.9
	<i>ns</i>	5.7	34.1	65.6	100.1	70.3	55.1
	<i>w</i>	4.6	25.1	58.9	45.3	25.5	31.9
	<i>ws</i>	4.5	27.2	48.6	30.2	25.9	27.3
	<i>wn</i>	5.5	34.1	83.1	145.5	108.7	75.4
	<i>wns</i>	5.0	27.4	85.1	130.8	78.3	65.3
	Mean	5.19	28.89	64.62	83.47	52.43	47.17
Kleinwanzleben	(1)( <i>ns</i> )	5.4	45.6	115.7	194.0	103.1	92.8
	<i>w</i> ( <i>ns</i> )	5.9	49.4	122.2	177.9	93.8	89.8
	Mean	5.64	47.32	118.95	185.95	98.50	91.31
<i>Treatment effects (mean of all leaves)</i>							
Hilleshög: Mean		47.17	Kleinwanzleben: Mean		91.31		
Main effects	<i>W</i>	+5.59*	Main effects		<i>W</i>	-2.69	
	<i>N</i>	+34.06*					
	<i>S</i>	-5.28*					
			S.E. 8.26				
Interactions	<i>WN</i>	+11.70*					
	<i>WS</i>	-2.06					
	<i>NS</i>	-2.64					
	S.E.	2.03					

It is evident that there is very considerable variation in the number of cells in the leaf and that the nitrogen supply profoundly affects the number of cell divisions occurring in the leaf initial. The cell number per leaf increases up to the 10th leaf in the low-N series and up to the 15th leaf in the high-N series. This general increase in cell number depends on the ontogenetic succession of the leaves. The falling off after the 10th or 15th leaf is undoubtedly due to lack of nitrogen, as is shown by comparison with fully manured field material in which much later leaves than the 15th show cell numbers in the range of 150 to 250 with occasional values of 300 to 320, which seem to represent the maximum number for sugar-beet. In this experiment the nitrogen supply, even in the high-N series, must have been limiting growth by about the beginning of the second growth period (middle of July). This is confirmed by the decreased rate of formation of new leaves in the second growth period. The additional nitrogen given to the high-N series was 1 g. per pot; in the cultural conditions employed an addition of about 3 g. N per pot would be required to maintain optimal growth according to general experience with sugar-beet.

The effect of the higher level of nitrogen in increasing the number of cell divisions is very marked in both high- and low-water series. Even in the first leaf there is an indication of an effect of nitrogen, although the first leaf

initial is formed so early that it might be expected to be unaffected by the external supply of nutrients. In Hilleshög low water-supply causes a definite reduction in the number of cells in the leaf and the reduction is more marked in the high-N plants. The effect of water régime is not very large, however, and is not entirely consistent in the individual leaves. In Kleinwanzleben water régime is without effect on cell number. It is possible that in both varieties there might be a greater effect at higher N levels. Salt causes a slight reduction in cell number which is consistent for all leaves.

The effect of alternation of the water régime on cell number can only be judged by reference to the 20th leaf. Although the 15th leaf appeared after the alternation, in most plants it must have been formed at about the time the change was made and therefore reflects the earlier water régime. Cell numbers for the 20th leaf are given in Table VII. Change of water régime does not cause any significant change in cell number, and this is consistent with the relatively small effect of water-supply already noted.

TABLE VII  
*Mean Number of Cells per Leaf (in hundred thousands) in the  
20th Leaf (Sugar-beet)*

Treatment.		Mean number of cells per leaf.	Mean	
				53.51
Hilleshög	(1)	19.1	Main effects	$W_1$ +10.41
	<i>s</i>	18.4		$W_2$ +3.96
	<i>n</i>	73.2		$N$ +69.69*
	<i>ns</i>	70.3		$S$ -5.89
	$w_1$	17.9	Interactions	$W_1 W_2$ -2.15
	$w_1 s$	12.6		$W_1 N$ +6.77
	$w_1 n$	114.8		$W_2 N$ +0.61
	$w_1 ns$	85.9		$W_1 S$ -10.17
				$W_2 S$ +3.31
	$w_2$	16.8		$NS$ -3.54
	$w_2 s$	13.1		
	$w_2 n$	75.5		S.E. 7.46
	$w_2 ns$	100.0		
	$w_1 w_2$	25.5		
	$w_1 w_2 s$	25.9		
	$w_1 w_2 n$	108.7		
	$w_1 w_2 ns$	78.3		
Kleinwanzleben	(1)( <i>ns</i> )	103.1	Mean	99.67
	$w_1$ ( <i>ns</i> )	85.2	Main effects	$W_1$ -20.23
	$w_2$ ( <i>ns</i> )	116.4		$W_2$ +10.94
	$w_1 w_2$ ( <i>ns</i> )	93.9	Interactions	$W_1 W_2$ -2.33
				S.E. 23.59

### Cell size

In determining the number of cells per leaf, a figure for the number of palisade cells per unit area is obtained by a direct count on the leaf when cell expansion is sensibly complete. This figure is a rough inverse measure of cell size. It is only a rough measure because it takes no account of variations

in the proportion of intercellular space. This is the only estimate of cell size available, since it was impossible to measure the large number of individual cells necessary to get a more precise estimate. The information relating to cell size is summarized in Table VIII.

TABLE VIII  
*Number of Cells per Unit Area (0.000365 sq. cm.) in Leaves  
of Sugar-beet (means)*

		Constant water régime.					Alternation of water régime.	
	Treatment.	1st leaf.	5th leaf.	10th leaf.	15th leaf.	20th leaf.	Treatment.	20th leaf.
Hilleshög	(1)	6.4	12.1	32.9	66.9	75.8	$w_1$	85.7
	$s$	5.7	11.3	31.4	82.8	104.7	$w_1 s$	72.0
	$n$	6.8	12.5	22.7	58.7	51.8	$w_1 n$	72.5
	$ns$	5.9	12.8	14.6	42.2	47.2	$w_1 ns$	45.2
	$w_1 w_2$	5.5	11.5	30.9	69.8	83.0	$w_2$	78.0
	$w_1 w_2 s$	4.5	11.8	34.2	60.5	89.0	$w_2 s$	73.2
	$w_1 w_2 n$	5.9	10.3	20.7	53.0	56.6	$w_2 n$	32.0
	$w_1 w_2 ns$	5.6	8.4	15.9	42.8	76.5	$w_2 ns$	45.2
Kleinwanzleben	(1)( $ns$ )	5.8	23.8	23.7	60.4	53.4	$w_1(ns)$	67.6
	$w_1 w_2(ns)$	5.4	23.1	23.1	58.2	66.9	$w_2(ns)$	55.3

*Treatment effects on cell size*

(+ denotes increase, — denotes decrease, in cell size)

Hilleshög	$W_1 W_2$	+	+	±	+	—	$W_2$	+
	$N$	—	+	+	+	+	$N$	+
	$S$	+	±	+	+	—	$S$	+
Kleinwanzleben	$W_1 W_2$	+	+	+	+	—	$W_2$	+

The higher level of nitrogen consistently increases the final cell size. Low water-supply tends to decrease cell size, especially at the higher nitrogen level, but the results are not entirely consistent. Salt tends to increase cell size, in some cases quite considerably, but the effects are again not altogether consistent. Variation in cell size between replicates is often very large and the figures can only be used to indicate general trends in cell sizes. Since the phase of cell extension must be closely dependent on water-supply, it might be expected that the low-water plants would show greater variation in cell size than the high-water plants because, in the former, leaf expansion may coincide with a wide range of water-levels from saturation to extreme drought. Variation between replicates is, however, almost the same in both high- and low-water series. Taking the 15th and 20th leaves, for example, the standard deviation in the high-water series is 15.8 per cent. and in the low-water series 18.7 per cent. It appears that some uncontrolled factor is operating on the phase of leaf expansion. Light is known to exert considerable influence on this phase and is therefore a possible disturbing factor. A more probable source of variation is the limited amount of soil from which, in pot culture, absorption is possible. Thus even in the high-water series the absolute

amount of water available is relatively small and when leaf expansion is active there may be quite sharp fluctuations in the water-level, leading to considerable variations in the final cell size.

In this connexion a comparison of cell size in experimental pot plants and sugar-beet grown in the field is of some interest. The field material was Kleinwanzleben from commercial seed and therefore not closely comparable with the selected Kleinwanzleben used in the experiment. Field material showed rather less variation in cell size between comparable leaves than pot plants and no marked effects due to fertilizer treatments. The mean number of cells per unit area in the 15th leaf was 31.5, which may be compared with 58.2 in the 15th leaf of experimental Kleinwanzleben with high water-supply and added nitrogen and salt. Later leaves of field material have in general larger cells than the 15th and 20th leaves of the experimental plants. Whilst the reduction in cell size in the later leaves of the pot plants may be partly due to depletion of nitrogen, there seems to be an indication of another factor associated with pot culture which tends to reduce cell size. This factor is probably either the limited amount of water available in the pot or a relative reduction in root development due to growth in a restricted volume. There is further evidence from the effects of defoliation, to be discussed later, which suggests that, in small pots at least, water-supply may be limiting leaf expansion (cell size) even in fully watered plants.

The importance of the phase of leaf expansion as a determinant of leaf area is well illustrated by the following comparison between two leaves, both measured on the same day (August 15), which both have a high total number of cells per leaf (approaching what appears to be the maximum). The first leaf is a 15th leaf taken from the pot experiment, the second is the largest leaf that was found on a fully manured field plant.

Leaf.	Treatment.	No. of cells per leaf (in hundred thousands).	No. of cells per unit area.	Area (cm. <sup>2</sup> ).
Experimental pot culture	<i>w<sub>2</sub>(ns)</i>	253.9	55.5	167
Field	fully manured	317.1	17.1	675

The present experimental work indicates the complexity of the relations between cell expansion and internal and external conditions, and emphasizes the need for more detailed investigation of this aspect of leaf growth.

#### *Area of individual leaves*

Table IX shows the changes in the area of individual leaves; these are the summation of effects on cell size and number of cells per leaf which have already been analysed.

#### *Growth and yield*

Material from the sugar-beet experiment was harvested on three occasions which provided the opportunity to obtain data on the course of growth. The first harvest was on May 30 when the plants were thinned from 2 to 1 plant

TABLE IX  
*Mean Area of Successive Leaves of Sugar-beet (cm.<sup>2</sup>)—  
 Constant Water Régime*

	Treatment.	1st leaf.	5th leaf.	10th leaf.	15th leaf.	20th leaf.	Mean.
Hilleshög	(1)	28.6	71.2	63.2	30.4	9.5	40.58
	<i>s</i>	32.2	78.7	61.7	24.1	6.5	40.64
	<i>n</i>	32.8	98.4	114.3	74.2	51.5	74.24
	<i>ns</i>	35.9	101.6	134.2	89.3	60.7	84.34
	<i>w</i>	31.4	78.7	62.7	22.6	11.3	41.36
	<i>ws</i>	37.4	84.0	48.6	18.6	10.7	39.86
	<i>wn</i>	35.0	116.5	145.8	100.7	69.3	93.46
	<i>wns</i>	34.0	124.2	187.3	115.9	37.8	99.84
Kleinwanzleben	(1)( <i>ns</i> )	41.4	129.7	177.6	123.5	71.5	108.74
	<i>w(ns)</i>	39.8	160.0	195.9	112.0	51.3	111.80

*Treatment effects  
 (Mean of all leaves)*

Hilleshög: Mean	64.31	Kleinwanzleben: Mean	110.27
Main effects		Main effects	
<i>W</i>	+8.67	<i>W</i>	+3.06
<i>N</i>	+47.36*	S.E.	10.54
<i>S</i>	+3.76		
Interactions			
<i>WN</i>	+8.68		
<i>WS</i>	-1.81		
<i>NS</i>	+4.47		
S.E.	4.54		

per pot. The thinnings were removed as completely as possible and treated as replicates of the plants left growing. The second harvest was on July 17 when three blocks were harvested. The remaining three blocks were harvested on September 9 at the end of the experiment. The second harvest (July 17) divided the recorded experimental period into two almost equal periods of 7 weeks.

At each harvest the fresh weight and dry weight of the leaves (lamina), petioles (including crown), tap-root, and fibrous roots were recorded separately for each plant, and the total leaf area per plant was determined (for methods see Watson, D. J., 1937, 1947). The tap-root was analysed for sucrose and hexose in July and September but not at the first harvest (for analytical procedure see van der Plank, 1936; Harding and Downs, 1933). Total nitrogen was estimated by the Kjeldahl procedure in the material of the final harvest only.

Only the most important aspects of the bulky primary data can be summarized. The effects of the different treatments on the dry-matter yield at the final harvest are shown in Table X.

The most pronounced effect on yield of the whole plant is the increase caused by the higher nitrogen-level. High water-supply also increases yield, and this effect is more marked in the second than in the first half of the

TABLE X  
*Dry-matter Accumulation (g.) in Sugar-beet at Final Harvest—  
 Treatment Effects (means per plant)*

	Lamina.	Petiole.	Tap-root.	Fibrous root.	Whole plant.
Hilleshög					
Mean . . .	6.19	8.24	35.84	1.22	51.48
Main effects:					
$W_1$	-0.46	-0.27	+4.84*	+0.17	+4.28
$W_2$	+0.54	+1.04	+11.19*	-0.25	+12.52*
$N$	+5.04*	+8.51*	+29.67*	+0.59*	+43.81*
$S$	+0.26	+0.32	+0.96	+0.03	+1.57
Interactions:					
$W_1 W_2$	-0.27	-0.22	+0.30	-0.25	-0.44
$W_1 N$	-0.33	-0.04	+3.46	+0.11	+3.20
$W_2 N$	+0.16	+0.68	+7.46*	+0.17	+8.47*
$W_1 S$	+0.01	+0.79	-0.08	+0.03	+0.75
$W_2 S$	+0.30	-0.27	+2.93	-0.04	+2.82
$NS$	+0.36	+0.65	-0.19	-0.14	+0.68
S.E.	0.26	0.61	1.90	0.14	2.21
Kleinwanzleben					
Mean . . .	9.03	14.94	52.48	2.44	78.89
Main effects:					
$W_1$	-2.28*	+0.25	-0.93	-0.62	-3.59
$W_2$	+0.78	+4.48*	+19.23*	+0.18	+24.68*
Interactions:					
$W_1 W_2$	+0.32	-0.08	+1.60	-0.38	+1.45
S.E.	0.86	1.26	2.59	0.61	4.25

growth period. In the second period the effect of high water is significantly enhanced when associated with the higher level of nitrogen. The effects of salt are irregular and not statistically significant.

The effects of water-supply in the alternating and in the continuous series are quite consistent. The change from high to low water reduces the final yield but not to the level of the continuous low-water plants. The change from low to high water raises the final yield but not to the level of the continuous high-water series. (The most striking feature is the yield of the plants at the higher nitrogen level when changed from low to high water-supply. The yield is almost as high as in the corresponding plants with continuous high water-supply). This point is discussed in connexion with the nitrogen figures. When dry-matter accumulation is considered in the different parts of the plant separately, it is found that, whilst nitrogen increases the dry matter in all parts, the effects of water are only recorded in the dry matter of the tap-root and not significantly in lamina and petiole.

At each harvest the total leaf area of each plant harvested is obtained from the leaf-weight/leaf-area ratio of a sub-sample from the lamina material. At intervals between the harvests it is possible to estimate the total leaf area of the growing plants from the records of leaf number and leaf area and the

measurements of length and breadth of the leaves. Two estimates were made in each period (i.e. at intervals of 2 and 3 weeks).

The changes in total leaf area during growth may be seen from the figures in Table XI, which give a clearer picture than graphical representation. The principal effect on leaf area is clearly that of nitrogen in increasing the rate

TABLE XI  
*Mean Leaf Area per Plant (in square decimeters)*

Variety.	Treatment.	May 30.	June 11.	June 25.	July 15.	July 30.	Aug. 14.	Sept. 9.
Hilleshög	(1)	19	94	462	555	629	672	492
	<i>s</i>	18	143	528	632	645	574	455
	<i>n</i>	16	114	631	1,365	1,702	1,540	1,294
	<i>ns</i>	22	126	672	1,634	1,569	1,598	1,439
	<i>w</i> <sub>1</sub>	—	—	—	—	695	500	518
	<i>w</i> <sub>1</sub> <i>s</i>	—	—	—	—	722	545	472
	<i>w</i> <sub>1</sub> <i>n</i>	—	—	—	—	1,809	1,456	1,201
	<i>w</i> <sub>1</sub> <i>ns</i>	—	—	—	—	1,915	1,726	1,264
	<i>w</i> <sub>2</sub>	—	—	—	—	672	632	518
	<i>w</i> <sub>2</sub> <i>s</i>	—	—	—	—	688	719	648
	<i>w</i> <sub>2</sub> <i>n</i>	—	—	—	—	1,599	1,678	1,411
	<i>w</i> <sub>2</sub> <i>ns</i>	—	—	—	—	1,895	1,950	1,733
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub>	20	113	425	601	795	710	585
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> <i>s</i>	18	86	428	577	683	691	604
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> <i>n</i>	20	169	735	1,685	2,098	2,068	1,315
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> <i>ns</i>	21	166	997	2,043	2,070	1,813	1,407
Kleinwanzleben	(1)( <i>ns</i> )	20	118	637	1,561	2,207	1,960	1,573
	<i>w</i> <sub>1</sub> ( <i>ns</i> )	—	—	—	—	1,576	1,386	1,090
	<i>w</i> <sub>2</sub> ( <i>ns</i> )	—	—	—	—	2,194	2,387	1,965
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> ( <i>ns</i> )	19	134	935	1,793	2,139	2,068	1,381

of formation of leaf initials combined with an additional effect on the size of the individual leaves. In all treatments leaf area begins to decrease more or less rapidly at about the beginning of August, doubtless owing to the exhaustion of the available nitrogen. The presence of salt appears to result in the more rapid utilization of nitrogen so that maximum leaf area is attained earlier; otherwise the effect of salt is small. In Hilleshög the effect of low water-supply in reducing leaf area (due to reduction of individual leaf size) is clearly seen in the high-N treatments. Leaf area in Kleinwanzleben follows essentially the same course as in the similarly treated plants of Hilleshög, except that an effect of water régime is shown only in the alternating series.

The sharp fall in leaf area when plants of the high-water series are switched over to low water-supply seems to be due to the accelerated senescence of a number of the older and larger leaves combined with the general fall due to nitrogen shortage. The fall in leaf area is delayed when plants are switched from low to high water-supply. This is partly due to a delay in senescence of the older leaves and (in the high-N series at least) to an increase in the

rate of formation of new leaves related to the internal nitrogen supply (see below).

From the data for dry-matter accumulation and leaf area the net assimilation rate (N A R) for plants of each treatment can be calculated, and these results are given in Table XII. Since the two growth periods are equal within a few days the net assimilation rate (N A R) over the whole period is the mean of the rates in the separate periods.

TABLE XII

*Net Assimilation Rate in Sugar-beet (as g. dry matter per  
sq. dm. leaf area per week)*

Variety.	Treatment.	N A R	
		May 30–July 17.	July 17–Sept. 9.
Hilleshög	(1)	0·684	0·333
	s	0·570	0·258
	n	0·594	0·343
	ns	0·572	0·295
	w <sub>1</sub>	—	0·335
	w <sub>1</sub> s	—	0·371
	w <sub>1</sub> n	—	0·318
	w <sub>1</sub> ns	—	0·220
	w <sub>2</sub>	—	0·343
	w <sub>2</sub> s	—	0·364
	w <sub>2</sub> n	—	0·498
	w <sub>2</sub> ns	—	0·464
	w <sub>1</sub> w <sub>2</sub>	0·657	0·297
	w <sub>1</sub> w <sub>2</sub> s	0·662	0·513
	w <sub>1</sub> w <sub>2</sub> n	0·643	0·421
	w <sub>1</sub> w <sub>2</sub> ns	0·688	0·370
Kleinwanzleben	(1)(ns)	0·428	0·253
	w <sub>1</sub> (ns)	—	0·309
	w <sub>2</sub> (ns)	—	0·439
	w <sub>1</sub> w <sub>2</sub> (ns)	0·619	0·430

The most pronounced general effect on N A R is clearly that of water-supply. The low-water level depresses N A R throughout the growth period in all plants except those which received neither salt nor nitrogen. In the latter, low water-supply either does not affect N A R or causes a slight increase. The average effect of continuous low water-supply over all treatments is to reduce N A R by about 15 per cent. In the plants with alternating water-supply the effect of low water-supply on N A R is only marked in the high-N plants.

Salt appears to be a factor influencing N A R although the effects are somewhat complex. In conjunction with low water-level salt distinctly reduces N A R. With high water-supply salt tends to increase N A R at the low level of nitrogen, but at the higher level of nitrogen the effects are small and doubtful.

It appears at first sight that nitrogen supply is without influence on N A R. Taking all treatments in the continuous series, the mean N A R over the whole period is exactly the same for high-N and low-N plants (0.496 g./dm.<sup>2</sup>/wk.). When the plants with alternating régime are considered, however, there is clear evidence of the effect of nitrogen on N A R, as is shown by the very considerable increase in N A R of high-N plants when switched from low to high water-supply. This is connected with the relatively high internal nitrogen content of the plants of the dry series.

The results of the nitrogen determinations on the material at the final harvest must now be briefly considered. The essential information is summarized in Table XIII. Replicate samples were combined before analysis. The figures for total nitrogen per plant given in the last column of the table are calculated and are not true means since they are derived from estimations of total nitrogen in material obtained by mixing three replicate sub-samples from each treatment. The significance of these figures is sufficiently clear to be unaffected by the slight bias inherent in the method of calculation.

TABLE XIII  
*Total Nitrogen in Sugar-beet at Final Harvest*

Variety.	Treatment.	N as % dry matter				Total N per plant (g.) (calculated).
		Lamina.	Petiole.	Tap-root.	Fibrous roots.	
Hilleshög	(1)	2.31	1.01	0.63	1.55	0.27
	<i>s</i>	2.60	1.33	0.66	1.47	0.26
	<i>n</i>	3.67	1.46	0.92	2.33	0.86
	<i>ns</i>	3.43	1.61	0.99	2.02	0.87
	<i>w</i> <sub>1</sub>	2.44	1.19	0.63	1.78	0.27
	<i>w</i> <sub>1</sub> <i>s</i>	2.38	1.03	0.56	1.41	0.25
	<i>w</i> <sub>1</sub> <i>n</i>	3.21	1.32	0.79	1.96	0.79
	<i>w</i> <sub>1</sub> <i>ns</i>	3.09	1.32	0.82	1.85	0.82
	<i>w</i> <sub>2</sub>	2.20	1.01	0.63	1.70	0.23
	<i>w</i> <sub>2</sub> <i>s</i>	2.48	1.03	0.56	1.45	0.28
	<i>w</i> <sub>2</sub> <i>n</i>	3.09	1.22	0.74	1.78	0.86
	<i>w</i> <sub>2</sub> <i>ns</i>	2.98	1.18	0.71	1.59	0.91
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub>	2.44	1.07	0.61	1.60	0.27
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> <i>s</i>	2.61	1.16	0.50	1.36	0.29
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> <i>n</i>	3.27	1.23	0.72	1.63	0.90
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> <i>ns</i>	2.71	1.13	0.65	1.52	0.84
Kleinwanzleben	(1)( <i>ns</i> )	3.28	1.43	1.00	1.89	0.99
	<i>w</i> <sub>1</sub> ( <i>ns</i> )	3.22	1.21	0.77	1.78	0.75
	<i>w</i> <sub>2</sub> ( <i>ns</i> )	3.03	1.06	0.68	1.54	0.96
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> ( <i>ns</i> )	2.93	1.02	0.58	1.51	0.81

It is evident that the uptake of nitrogen per plant has been as high in the dry as in the wet series. Since the plants of the dry series are smaller, the internal concentration of nitrogen is very considerably higher, especially in the high-N treatments. This is even more clearly seen if the nitrogen

is expressed on a fresh-weight basis. The relatively greater uptake of nitrogen from dry soil has been observed by other workers (Petrie and Arthur, 1943).

It may be noted that of the 1 g. of N added in the high-N series 60–70 per cent. is recovered in the plant.

High relative uptake of nitrogen in the dry series is accompanied by lowered N A R and relative lowering of the carbohydrate level in consequence. The supply of carbohydrate is presumably sufficient to provide for the conversion of the absorbed ammonia nitrogen into amides or other simple nitrogen compounds, but insufficient to metabolize these compounds to protein. The low-water plants therefore possess an internal store of readily available nitrogen. The effect of increasing the water-supply is in the first place to permit increased synthesis of carbohydrate which results in renewed protein synthesis expressing itself in the renewal of leaf production at the apex and the increase in yield which have been already mentioned.

The marked effect of nitrogen on N A R in these conditions suggests that N A R may be closely related to protein content. Crowther (1934) has pointed out that owing to the effect of nitrogen on growth the internal nitrogen content is, in a sense, self-regulating and tends towards a constant level characteristic of the species. This would explain the apparent lack of any effect of nitrogen on N A R since in both low- and high-N plants the internal nitrogen level, or perhaps more correctly the protein level, may not vary widely, except in the special conditions where alteration in level of water-supply creates temporarily an unusually high internal concentration of nitrogen. In this connexion it seems possible that the fall in N A R in the second half of the growth period may be associated with a falling internal nitrogen level as the supply of nitrogen in the soil is exhausted.

The results of the analyses for sucrose and reducing sugars are reported in Table XIV. Since the sugars form between 67 and 75 per cent. of the dry matter of the tap-root, the total sugar per plant is closely correlated with yield and shows the same general response to factors. The percentage of sugars on dry matter is consistently higher in plants of the wet than in plants of the dry series. This difference is presumably related to the higher N A R in the former.

Finally, a word may be said about the respective contributions of N A R and leaf area to yield in the given experimental conditions. By far the largest effect on yield is due to nitrogen, and this is almost entirely due to increase in leaf area. This increase results from the dual effect of nitrogen in accelerating the rate of production of leaves by the apical meristem and in increasing the mean area of individual leaves through its influence on cell division in the leaf initial. Nitrogen does not affect N A R except in the unusual conditions resulting from the abrupt change from low to high water régime. Salt affects both N A R and leaf area in a somewhat complex manner, but the net effects on yield are small and not significant.

TABLE XIV  
*Sucrose and Reducing Sugar in Tap-root of Sugar-beet at  
 Final Harvest (as % dry matter)*

Variety.	Treatment.	Sucrose.	Reducing sugar.
Hilleshög	(1)	71.7	0.52
	<i>s</i>	72.0	0.63
	<i>n</i>	71.7	0.88
	<i>ns</i>	72.4	0.70
	<i>w</i> <sub>1</sub>	70.0	0.44
	<i>w</i> <sub>1</sub> <i>s</i>	73.3	0.35
	<i>w</i> <sub>1</sub> <i>n</i>	70.7	0.43
	<i>w</i> <sub>1</sub> <i>ns</i>	73.9	0.66
	<i>w</i> <sub>2</sub>	69.6	0.45
	<i>w</i> <sub>2</sub> <i>s</i>	72.1	0.48
	<i>w</i> <sub>2</sub> <i>n</i>	70.2	0.46
	<i>w</i> <sub>2</sub> <i>ns</i>	74.2	0.66
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub>	75.3	0.50
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> <i>s</i>	73.5	0.41
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> <i>n</i>	72.2	0.87
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> <i>ns</i>	74.6	0.65
Kleinwanzleben	(1)( <i>ns</i> )	67.9	0.63
	<i>w</i> <sub>1</sub> ( <i>ns</i> )	68.7	0.59
	<i>w</i> <sub>2</sub> ( <i>ns</i> )	69.3	0.53
	<i>w</i> <sub>1</sub> <i>w</i> <sub>2</sub> ( <i>ns</i> )	67.5	0.46

#### EXAMINATION OF FIELD MATERIAL

A selection of field material grown with various manurial treatments was examined for comparison with the pot experiment. Most of this material was from the 'classical' experiments at Rothamsted. The general principle adopted was to take ten random samples of corresponding leaves from each manurial treatment. The area of each leaf was determined by means of a planimeter and a sample was taken for cell count for estimation of the number of cells in the leaf. The cell count was carried out in the same manner as for sugar-beet. The results for each material will be briefly reported together with details of sampling.

1. *Mangold and sugar-beet.* Samples were taken from ten treatments (from the Barnfield experiment) on August 22 (Table XV). The 15th leaf was taken from each of ten plants on each plot.

The main effects of the treatments are expressed as percentage increase or decrease (—) on the mean.

2. *Potato.* This material was taken from a large field experiment designed to compare four times of planting with four manurial treatments in factorial combination (Dung, N, P, K). Samples were taken on July 10 from the first planting only, which was then beginning to flower. Ten random samples were taken from each of the sixteen manurial treatments (Table XVI). To ensure corresponding material the penultimate leaflet from the 4th leaf below the flower on main shoots was taken.

TABLE XV

Manurial addition.	Mean area (cm. <sup>2</sup> ).	Mean no. cells per leaf (in hundred thousands).	Mean no. cells per unit area.
<i>Mangold</i> (15th leaf)			
(1)	50.28	38.9	28.50
<i>d</i>	171.35	104.5	23.50
<i>dpk</i>	111.76	59.8	22.28
<i>pk(s)</i>	67.11	38.3	26.00
<i>pk</i>	54.48	38.7	29.60
<i>n</i>	63.58	65.5	30.55
<i>nd</i>	258.00	132.2	19.96
<i>ndpk</i>	220.02	122.3	23.32
<i>npk(s)</i>	130.82	79.7	21.64
<i>npk</i>	177.79	107.0	21.53
<i>Sugar-beet</i> (15th leaf)			
(1)	43.74	37.0	32.36
<i>dpk</i>	149.90	101.4	26.78
<i>pk(s)</i>	60.76	66.1	39.21
<i>pk</i>	48.08	34.7	30.96
<i>n</i>	54.84	78.0	36.90
<i>ndpk</i>	123.50	84.7	23.45
<i>npk(s)</i>	104.48	86.0	31.56
<i>npk</i>	121.93	114.2	31.25

Treatments: *d* = 14 tons dung per acre.

*n* = 4 cwt. sulphate of ammonia per acre.

*pk* = 3½ cwt. superphosphate and 4½ cwt. sulphate of potash per acre.

*s* = 2 cwt. sodium chloride and 2 cwt. sulphate of magnesia per acre.

*Treatment effect (%) on*

Treatment.	Leaf area.	No. of cells per leaf.	No. of plots involved in comparison.
<i>Mangold</i>			
<i>D</i>	75	51	8
<i>N</i>	60	58	10
<i>PK</i>	4	—4	8
<i>S</i>	—16	—21	4
<i>Sugar-beet</i>			
<i>D</i>	46	22	4
<i>N</i>	29	41	8
<i>PK</i>	53	26	4
<i>S</i>	4	2	4

It must be noted that the results for potato refer to leaflets and not to whole leaves (which are difficult to measure owing to their irregular pinnate form). Although it is reasonable to assume that changes in the leaflet reflect similar variations in the whole leaf, there are no actual data to support such an assumption.

TABLE XVI  
*Potato (penultimate leaflet)*

Treatment.	Mean leaf area (cm. <sup>2</sup> ).	Mean no. cells per leaf (in hundred thousands).	Mean no. cells per unit area.
(1)	13.1	27.1	76.7
<i>d</i>	22.4	46.2	77.5
<i>n</i>	15.1	30.7	74.2
<i>dn</i>	23.3	48.2	77.5
<i>p</i>	15.2	30.5	73.3
<i>dp</i>	23.3	47.7	73.7
<i>np</i>	18.0	36.3	71.8
<i>dnp</i>	24.5	48.9	72.7
<i>k</i>	15.0	30.4	73.9
<i>dk</i>	22.7	43.3	70.3
<i>nk</i>	21.2	39.9	68.8
<i>dnk</i>	23.2	45.3	71.1
<i>pk</i>	16.8	32.7	71.1
<i>dpk</i>	21.1	44.0	76.7
<i>npk</i>	27.0	54.2	74.1
<i>dnpk</i>	21.8	44.5	74.9

Treatments: *d* = 15 tons dung per acre.*n* = 0.6 cwt. N per acre as sulphate of ammonia.*p* = 0.6 cwt. P<sub>2</sub>O<sub>5</sub> per acre as superphosphate.*k* = 1.0 cwt. K<sub>2</sub>O per acre as muriate of potash.

## Treatment effects (%) on

Treatment.	Leaf area.	No. of cells per leaf.
<i>D</i>	25	26
<i>N</i>	14	15
<i>P</i>	8	7
<i>K</i>	6	8

3. *Barley*. This material was collected on July 3 from ten treatments (from the Hoosfield experiment). Ten random samples were taken from each plot, taking the first leaf below the flag leaf from main shoots only. Although the leaf structure is very different from the leaves previously examined, it is possible to count the number of mesophyll cells by employing the technique already described and examining the upper surface of the leaf between the longitudinal bands of sclerenchyma. Cell counts were not, however, carried out on all the barley samples since examination of a small number of samples from each treatment showed no effects of treatment on the number of cells per unit area. The total number of cells per leaf can therefore be taken as closely proportional to leaf area (Table XVII).

The results with mangold, sugar-beet, potato, and barley, limited though they are, indicate clearly enough that the response of leaf growth to manurial and other factors is not necessarily uniform for all species. In mangolds and sugar-beet there is evidence of quite wide variation in both cell size and number of cells per leaf caused by manurial treatment. Nitrogen and dung

TABLE XVII  
*Barley (1st leaf below flag leaf)*

Treatment.	Mean leaf area (cm. <sup>2</sup> ).
(1)	16.63
(1)	16.51
<i>p</i>	16.13
<i>k</i>	17.08
<i>pk</i>	16.34
<i>n</i>	18.23
<i>np</i>	16.80
<i>nk</i>	20.18
<i>npk</i>	16.22
<i>d</i>	24.80

Treatments: *n* = 2 cwt. sulphate of ammonia per acre.

*d* = 14 cwt. dung per acre.

*p* = 3½ cwt. superphosphate per acre.

*k* = 2 cwt. sulphate of potash per acre.

Treatment.	Treatment effect (%) on leaf area.	No. of plots compared.
<i>N</i>	7.6	8
<i>P</i>	-9.6	8
<i>K</i>	2.9	8
<i>D</i>	21.0	2

cause a considerable increase in the number of cells per leaf in both species, and in mangold nitrogen and dung also increase cell size. In sugar-beet, however, whilst dung increases cell size, nitrogen either has little effect or decreases cell size. The effect of salt is in marked contrast in the two species.

In potato, manurial treatment appears to be without effect on cell size. The range of variation in cell number per leaf is much less than in sugar-beet or mangolds, but nitrogen and dung cause definite increase in cell number. The effect of nitrogen is greater in combination with P or K or both, whilst the interaction of dung with all other factors or combinations of factors is negative.

In barley neither cell size nor number of cells per leaf is significantly affected by treatment, except possibly by dung. The lack of any nitrogen effect is rather surprising in view of the fact that leaf growth takes place in barley, as in many monocotyledons, by means of a persistent basal meristem. It is strange that the temporary meristem or the leaf initial in sugar-beet, mangold, and potato should be stimulated to more prolonged activity by increased nitrogen supply whilst the more persistent leaf meristem of barley is not. Possibly the explanation lies in hormonal control of leaf growth by the apex which limits the growth of the leaf initial as the distance between it and the apex is increased. In leaves with a persistent meristem, the limiting factor to their growth during most of their life may be hormonal, and in this case any nitrogen effect would be very small in the mature leaf as it would only be shown in the brief initial phase of growth when the leaf was close

to the apex. The leaf areas quoted above for barley refer to the leaf blade only and it might be thought that inclusion of the leaf sheath would alter the picture. Measurements of the length of the leaf sheath were therefore made at the time that the leaf-blade samples were taken. Assuming that the area of the sheath is proportional to its length, the results showed no effects of treatment and no reason to modify the conclusions drawn from examination of the leaf blades.

These preliminary observations emphasize the need for a much wider survey of leaf material from the field. This would provide the data for a general picture of the effect of manurial and other factors on the main phases of individual leaf growth, cell division, and cell extension. Such a survey would have to take particular account of variations in response due to ontogenetic, climatic, and seasonal factors within each species, and would probably greatly alter the tentative ideas which have been outlined above.

#### LIGHT AND LEAF DEVELOPMENT

A preliminary and somewhat crude experiment on the effect of light on leaf development is worth reporting briefly as an indication of problems requiring investigation. Sugar-beet, variety Hilleshög, was sown on May 17 in small pots and thinned at an early stage to 1 plant per pot. Each pot received 0.2 g. N at sowing and the same amount about 6 weeks later, together with an adequate quantity of P, K, and NaCl. On July 27, 16 plants were taken and divided at random into 4 groups of 4 plants each. All the plants were very similar in size, development, and number of leaves. One group was allowed to continue to grow in the light in the open as before. A second group was placed in the dark by simply covering each plant with a large pot. A third group was defoliated as completely as possible so that only the smallest unexpanded leaves around the apex remained. The defoliated plants then continued in the light. A fourth group was defoliated in the same way and placed in the dark.

On August 14 all dark plants were returned to the light. The dark plants had produced each 3-4 chlorotic leaves with enormously extended petioles and very small curled laminae. These leaves all died in a day or two when returned to light and could not be further examined. They would not be likely to show any effects on cell number, however, since these leaves must have been in the primordial stage at the time of transfer to darkness. The results demonstrate the profound effect of light on the phase of cell extension in the leaf. The differential effect on the cells of the petiole and those of the lamina is very striking. This effect of light is of course well known.

The 5th leaf produced in darkness only expanded after the plants were returned to the light. It can be fairly safely assumed that the initial of this leaf must have been produced in complete darkness. About 15 days after return to daylight, when expansion was complete, these leaves were examined and compared with the corresponding leaves produced by plants grown in the light (that is, with the normal daily periods of daylight). The results are

given in Table XVIII, from which there appears evidence that light may directly influence the number of cell divisions in the leaf initial. There does not seem to be any significant effect of defoliation on the number of cells per leaf. Although the number of cells per leaf is considerably reduced in the absence of light, the cells are clearly capable of expanding to their normal size as soon as the leaves are returned to light. Microscopic examination shows that even in the leaves most markedly reduced in area as a result of darkness the number of cell layers in the leaf remains unchanged.

TABLE XVIII

*Comparison of Corresponding Leaves, the Initials of which were produced respectively in Darkness or in Light*

		Mean leaf area (cm. <sup>2</sup> ).	Mean no. of cells per unit area.	Mean no. of cells per leaf (in hundred thousands).
Initials formed in light	Normal	14.3 ± 2.08	39.4 ± 2.01	15.3 ± 2.25
	Defoliated	24.1 ± 0.86	32.9 ± 1.92	21.7 ± 1.96
Initials formed in darkness	Normal	7.0 ± 1.90	32.0 ± 2.05	6.0 ± 1.53
	Defoliated	3.6 ± 0.88	33.8 ± 2.89	3.1 ± 0.68

Difference between mean number of cells per leaf in light and  
in darkness = 13.8 ± 1.95

There is an obvious effect of defoliation on cell size in the plants grown in the light. This effect is even more clearly observed in the earliest new leaves to expand after defoliation. The first new leaf of the defoliated plants is very much larger than the corresponding leaf of the plants not defoliated. As further new leaves are produced the difference in size between the defoliated and the non-defoliated plants becomes gradually less pronounced. It is clear from the figures in Tables XVIII and XIX that defoliation causes a very considerable increase in cell size in the leaves produced immediately afterwards. There is no effect on the number of cells per leaf or on the number of cell layers.

TABLE XIX

*Comparison of First New Leaf produced in Normal and Defoliated Plants of Sugar-beet*

		Mean leaf area (cm. <sup>2</sup> ).	Mean no. of cells per unit area.	Mean no. of cells per leaf (in hundred thousands).
Normal	.	24.2 ± 2.78	52.80 ± 5.29	34.2 ± 2.78
Defoliated	.	63.2 ± 3.73	18.75 ± 2.02	32.2 ± 2.66
Difference	.	39.0 ± 4.65	34.05 ± 5.66	1.9 ± 3.85

The most probable explanation of the pronounced effect of defoliation on cell size is in terms of water-supply. It seems that even in fully watered plants (in pots at least) the supply of water to the expanding leaves may be

limited by the demands of the older leaves. The effect of defoliation on plants in the field has not been tested.

#### DISCUSSION

The significance of the results has already been briefly discussed when reporting them and it is only necessary to add a few words of comment. The results emphasize the conception of leaf production, in its most general aspect, as an expression of the activity of the apical meristem. Factors which stimulate the activity of the meristem lead to an increase in the rate of production of leaf initials. The rate of production of individual leaves is the most important factor in determining leaf area.

Nitrogen supply has a pronounced effect on meristematic activity and the formation of new leaves. This is clearly a direct effect on protein synthesis and cell division at the apex. The relative insensitivity of apical activity to water-supply is rather remarkable. Over a period of more than 3 months the rate of formation of new leaves was the same in plants exposed to severe and continuous drought as in fully watered plants, although drought caused a marked reduction in net assimilation rate and presumably in carbohydrate supply. The lack of any direct effect of drought on apical activity during the experimental period is probably in part explained by the capacity of the apex to withdraw water and carbohydrate preferentially from other parts of the plant. However, the internal concentration of nitrogen was higher in plants exposed to drought than in fully watered plants and, when the former were transferred to a high water régime, the rate of formation of new leaves was increased. It appears, therefore, that reduced water-supply does tend to reduce the activity of the meristem, although in the experimental conditions this tendency is counterbalanced by the higher internal nitrogen concentration associated with low water-supply. Nevertheless, synthetic activity at the apex would seem to be less easily disturbed by changes in water tension than in mature organs such as leaves where the balance between protein synthesis and hydrolysis is readily changed in the direction of hydrolysis by loss of water.

Whilst leaf area depends primarily on the number of individual leaves produced in a given period by the apical and sub-apical meristems, it also depends on the size attained by individual leaf members. The leaf initial is a temporary and limited meristem, and the duration and extent of its activity is dependent on external and internal conditions. The internal conditions, which may be genetic but are at present unknown, set an upper limit to the size of the leaf at a particular ontogenetic stage. Within these limits there may be considerable response to nutrient and other external conditions. The meristematic stage in the leaf initial of sugar-beet has been shown to be considerably affected by nutrient supply, especially nitrogen. Higher levels of nitrogen may increase the number of cells in corresponding leaves as much as three or four times the number at lower levels of nitrogen. These increases are reflected in the size of the mature leaves. Other nutrient factors have

similar although much smaller effects. The degree to which cell division in the leaf initial depends on nutrient supply varies for different plants, and some species show much less variation in cell number in the leaves in response to manurial treatment than sugar-beet. In such plants (e.g. barley) the stage of cell division in the leaf initial makes little if any contribution to variations in leaf area. The leaf in plants such as sugar-beet and mangold in which the activity of the leaf meristem shows marked response to nutrient conditions would appear to be favourable material for investigation of the factors which determine meristematic activity. This activity is recorded in the number of cells of the mature leaf and can be easily and quantitatively evaluated. An investigation of leaf growth along these lines should yield valuable information on the problems of differentiation and the persistence of meristematic activity. A significant result of the present work is the demonstration that light exercises its profound effect on leaf growth both at the stage of cell division and at the stage of cell extension.

The observations which have been made on cell extension (cell size) point to the importance of external conditions, especially water-supply and light, in determining this phase of leaf growth. Variation in the degree of cell extension may be considerable and may be significant in the determination of leaf area. The relations between cell extension and external conditions appear to be complex, and it is clear that fuller investigation is required.

#### SUMMARY

The effect of nitrogen, salt (sodium chloride), and water-supply on leaf growth was investigated in two varieties of sugar-beet grown in pot culture.

The rate of production of leaves by the apical meristem was increased by increasing the external nitrogen supply, but was unaffected by salt or water-supply in the experimental conditions.

The number of cells in the individual leaf of one variety was increased with increase in nitrogen and in water-supply but was decreased by application of salt. In the other variety water-supply had no effect on the number of cells in the leaf (nitrogen and salt not tested).

Cell size in the leaf showed evidence of considerable variation with external conditions, especially water-supply, but no conclusions can be drawn from the limited data.

The number of cells in the leaf is greatly reduced when the initials arise in complete darkness, although such leaves are capable of normal cell extension, if returned to light soon enough.

The effects of the treatments on yield and leaf area were examined. Net assimilation rate was found to be unaffected by external nitrogen supply, although there was some evidence that it is not independent of the internal nitrogen level. NAR is generally reduced by low water-supply and is affected in a complex manner by salt.

Examination of material from field crops shows that the extent to which cell number and cell size in the leaf are affected by manurial treatment may vary considerably with different species.

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# The Ovule and 'Seed' of *Araucaria Bidwilli* with Discussion of the Taxonomy of the Genus

## I. Morphology

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With Plate VI and twenty Figures in the Text

## INTRODUCTION

THE bunya-bunya, *Araucaria Bidwilli* Hook., since specimens were first obtained in 1843 from Queensland, Australia, has stood out among conifers in several ways and has received unusual attention among botanists. A timber tree of much value and a handsome ornamental tree in tropical and sub-tropical climates, it has cones and 'seeds' of extraordinary size and weight for those of conifers. Its ovulate cone scale has played a prominent part in the controversial problem of the nature of this structure in the Araucariaceae and in conifers generally. Some of its characters suggest that it is much the most primitive species among the living Araucariaceae, and in some anatomical characters a primitive living conifer.

Taxonomically it has been tossed about within the genus and its position there is still in question. It is transitional in some characters between the two established sections of the genus. Further, the description in recent years of three new species of *Araucaria*—*A. Klinkii* Lauterb., *A. Hunsteinii* K. Schum., and *A. Schumanniana* Warb. from New Guinea—of which at least the last two species resemble *A. Bidwilli* in important leaf characters—raises again the question whether the bunya-bunya correctly belongs in the section *Columbea*<sup>1</sup> with the South American species where it is at present placed.

The earliest account of *A. Bidwilli* is that of John Bidwill (1842), who published a short description of a new *Araucaria* from Queensland, Australia. The botanical description of the species by Hooker (1843) from specimens sent to England by Bidwill came in the following year. At that time only five other species of *Araucaria* were known. These were of two distinct types: the Western Hemisphere species, *A. araucana* (Molina) K. Koch and *A. angustifolia* (Bertol.) O. Ktze., with relatively large leaves, wingless cone-

<sup>1</sup> *Columbea* is the correct spelling. Endlicher's spelling '*Colymbea*' is an error. See discussion in second part of this paper to be published later.

scales, and hypogeal germination; and the Eastern Hemisphere species, *A. columnaris* (Forst.) Hook., *A. excelsa* (Lamb.) R. Br., and *A. Cunninghamii* Ait., with small leaves, winged cone-scales, and epigeal germination. *A. Bidwilli*, with its combination of relatively large leaves and winged cone-scales, appeared to be transitional between the two groups. Although in early years of botanical acquaintance with the araucarian conifers the two types were placed in separate genera, by the time *A. Bidwilli* was discovered, Jussieu's genus *Araucaria* had become established and the genus had been divided by Endlicher (1842, p. 26) into two subdivisions, 'Colymbea' and 'Eutacta'. Antoine (1840-7, p. 107), who divided the genus into subgenera, placed *A. Bidwilli*, about 1846, in his subgenus *Eutacta* with *A. excelsa*, *A. columnaris*, and *A. Cunninghamii*, but left it, however, unnumbered in his list. Endlicher (1847, p. 187), in his subsequent treatment of the genus, also placed *A. Bidwilli* with the Eastern Hemisphere species, which it seemed to resemble in its winged cone-scales. Gordon (1858, p. 22) was apparently the first to place *A. Bidwilli* in 'Colymbea' with the two South American species which it resembled in leaf characters, although its type of germination was apparently not then known. Dürre (1865) finally established the germination as hypogeal and the cotyledon number as two, characters likewise certainly columbean.

Subsequently *A. Bidwilli* has remained in the section *Columbea*, although it has been recognized as an anomalous member of that section (Pilger, 1926, p. 263). Not only its broadly winged cone-scales but its geographical distribution—Queensland, Australia—separates it from the living American species of *Columbea*. Evidence of a wider distribution for this transitional araucarian type exists, however, in the fossil *Proaraucaria mirabilis* (Speg.) Wieland (Wieland, 1935, p. 19) from the Cerro Cuadrado forests of Patagonia. In this fossil, which most closely resembles *A. Bidwilli* (Darrow, 1936), broad leaves are likewise combined with broadly winged cone-scale units in which, moreover, the fertile scale and bract show a still more marked separation from one another than do those of *A. Bidwilli*.

A closer study of ovule, seed, and scale characters at various ages in this species has been made possible by the kindness of Dr. Leo F. Hadsall who has made available to the authors collections from trees growing at Fresno, California, and by Dr. Cyril E. White, Government Botanist of Queensland.

Dr. White (1947) has recently formed a new section of *Araucaria*, *Intermedia*, in which he has placed the three New Guinea species. The critical study of cone-scale and 'seed' characters of *A. Bidwilli* shows the need for further consideration of the sectional treatment of the genus. This paper presents, in part, the morphological basis for a further modification of the sectional treatment which the authors will present in a succeeding paper.

#### MATURE CONES AND 'SEEDS'

The mature ovulate cones of *A. Bidwilli* grown in California measure up to 35 cm. in length by 20 cm. in diameter, weigh up to 12 lb., and contain as many as 150 seeds. The scale units are 8-10 cm. long by 7-8 cm. wide (Pl. VI,

Fig. 12). The 'seeds' are extremely large for those of conifers (Pl. VI, Figs. 1-11), ranging in length up to 5.5 cm. and in diameter to 3.5 cm., and appear deeply sunken in the cone scale.

When the cone is mature the scale units fall away one by one, as in other species, or the entire cone may fall and break up on the ground. The 'seeds', which commonly are freed from the scale units at this time, represent matured ovules plus accessory tissues of the fertile scale. In all other species, so far as yet known, the ripened seed remains attached to, but only apparently enclosed in, the tissues of the indehiscent cone-scale unit (fused bract and fertile scale) (Pl. VI, Figs. 13-16).

In the best-known conifers the dominant member of the two organs composing the ovulate cone unit is the fertile scale. In the family Araucariaceae the bract is most prominent; the fertile scale is greatly reduced, and in *Agathis* and some species of *Araucaria* it is obscure (Pl. VI, Figs. 13, 14). In other species of *Araucaria* it is prominent and obvious as a complete organ adnate for nearly its full length to the ventral surface of the fertile scale.

In the mature unit of *A. Bidwilli* (Pl. VI, Fig. 12; Text-figs. 15, 16) the bract (*br*) is large and woody, with a thick apophysis (*ap*) and an acuminate, leaf-like point. Lateral to the median 'seed' it is extended as broad woody wings (*w*). The fertile scale distal to the 'seed' is a prominent, thick, woody structure, about one-third the length and one-third the width of the bract. It is free from the bract at the tip and along the distal lateral margins. The huge inverted 'seed' occupies two-thirds the length and about half the width of the unit. Covering the 'seed' is a thin, brown, membranous layer (*i-1*) which is continuous with the surface layers of the fertile scale and bract. This delicate layer, usually broken and frayed when the cone is mature, alone holds the mature 'seed' within a deep depression in the unit (Pl. VI, Fig. 12). As the cone scales shrink in drying, the layer is further torn, and when the unit falls the 'seed' is freed.

The free 'seed' is roughly ovoid, with the upper side somewhat flattened, and has a heavy, light-coloured, stony coat (Pl. VI, Figs. 1-11). The micropyle is evident as a small, well-defined hole in the coat at the proximal end. When the scale is attached, the micropyle lies close against the cone axis. From the micropyle a lateral ridge (*r*) extends on each side about two-thirds the length of the 'seed' (Pl. VI, Figs. 5, 11; Text-fig. 8). These ridges seem to mark the lateral limit of fusion between integument and fertile scale (Text-fig. 10, A, B). They delimit dorsal and ventral surfaces of the 'seed'. From these, as well as from the form of the cone-scale unit itself and the ontogeny of the ovule, it is evident that the expansion of the developing 'seed' is greatest downwards, at the expense of the tissues of the fertile scale and bract below. These tissues are strongly compressed—in the dry state are only about 1 mm. thick (Text-fig. 16). Layers representing the two organs are not usually distinct, but on some 'seeds' a layer, apparently representing the fertile scale and its bundles, remains adherent to the dorsal surface of the ovule (Pl. VI, Figs. 3, 7; Text-fig. 8, *fs*). The 'seed' coat in these specimens is often

grooved and the scale-bundles lie in these grooves (Pl. VI, Figs. 8–11; Text-fig. 8, *vb*).

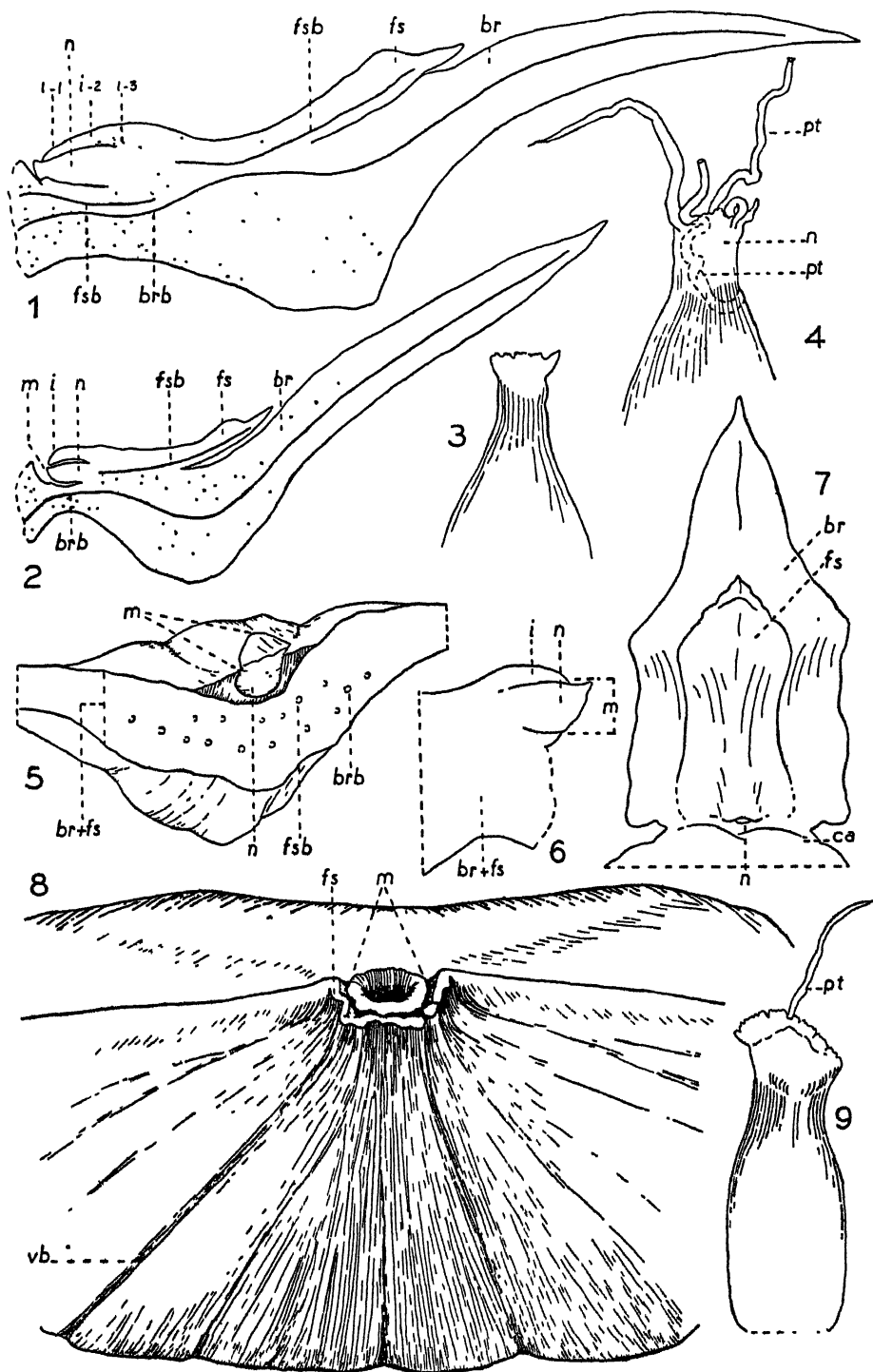
The appearance of the mature cone unit in all species of *Araucaria* bears out the usual taxonomic description of the genus (especially in contrast with *Agathis*) as having the ovule ‘embedded’ (‘enclosed’, ‘sunken’, ‘eingeschlossen’) in the cone scale. The setting free of an enclosed ‘seed’ in *A. Bidwilli* seems to support this view. But the ‘seed’ is not a true seed and there is no evidence that tissues of the fertile scale at any time ‘enclose’ the ovule. The ovule, like that of other conifers, lies on the ventral surface of the fertile scale and, as in some genera, is fused with it throughout its length. In ontogeny, tissue of the scale at no time surrounds the ovule. The ovule should not be described as enclosed; it is no more so than that of *Pinus* and other genera in which the ovule is adnate to the scale. Development of the ovule, in common with the scale and bract, forms a nut-like (Pl. VI, Figs. 13, 14) or samara-like (Pl. VI, Figs. 15, 16) fruiting structure in which the ovule only *appears* embedded.

#### ANATOMY OF CONE SCALE, OVULE, AND SEED

Early stages of cone-scale and ovule development were studied in longitudinal and transverse, hand-cut sections and compared with sections of mature scales and seeds to determine the nature of the seed-coat and the course of the vascular bundles.

In the youngest material of *A. Bidwilli* available (from native trees in Queensland) collected not long before pollination, the fertile scale is clearly visible as a structure adnate to the ventral surface of the bract (Text-fig. 2). It is about half as long as the bract, and is completely free from it for at least half its length; at the margins it is free nearly to the base. The ovule is a minute structure close to the scale axil, with well-differentiated nucellus (*n*) and integument (*i*). It lies definitely on the surface of the fertile scale. The micropyle (*m*) is large and open. In section (Text-fig. 2) no layering is yet visible in the integument, but some partly procambial vascular strands of bract and fertile scale are present.

TEXT-FIGS. 1–9. *A. Bidwilli*. Fig. 1. Diagrammatic longitudinal section of bract-scale unit and ovule three weeks after pollination. *br*, bract; *brb*, bract bundle; *fsb*, fertile-scale bundle; *n*, nucellus; *i-1*, outer layer of integument; *i-2*, median layer of integument; *i-3*, inner layer of integument. ( $\times 4$ .) Fig. 2. Diagrammatic longitudinal section of bract-scale unit and ovule shortly before pollination. *i*, integument; *m*, micropyle. ( $\times 5$ .) Fig. 3. Apical portion of nucellus of unfertilized ovule one year after pollination. ( $\times 12$ .) Fig. 4. Apical portion of nucellus of ovule at fertilization stage, one year after pollination, showing several pollen tubes (*pt*). ( $\times 12$ .) Fig. 5. Bract-scale unit, cut close to the cone axis, showing nucellus (*n*) projecting through micropyle (*m*) of ovule at pollination. *br+fs*, fused bract and fertile scale, showing cut ends of vascular bundles: (*brb*) of the bract; (*fsb*) of the fertile scale. ( $\times 12$ .) Fig. 6. Diagrammatic vertical longitudinal section of portion of bract-scale unit and ovule in Fig. 5, showing integument (*i*) and nucellus (*n*) projecting through micropyle (*m*) at pollination. ( $\times 12$ .) Fig. 7. Ventral view of bract-scale unit attached to cone axis (*ca*), showing separation of fertile scale (*fs*) and bract (*br*) 3 weeks after pollination. Nucellus (*n*) projects through micropyle. ( $\times 2$ .) Fig. 8. Micropylar view of mature seed showing portion of fertile scale (*fs*) still adherent to dorsal surface of seed; vascular bundles (*vb*) lie in grooves. ( $\times 5$ .) Fig. 9. Dorsal view of nucellus of ovule one month after pollination, showing blunt apical portion and ‘fringed’ margin penetrated by pollen tube (*pt*). ( $\times 12$ .)



FIGS. 1-9.

At pollination stage, in material collected at Fresno, California, slight enlargement has occurred (Text-figs. 5, 6). The micropyle (*m*) is a nearly round opening, somewhat deeper on the under side, through which is seen protruding the nucellus (*n*). About 3 weeks after pollination the fertile scale is more prominent and still free about half its length and at the margins nearly to the base (Text-fig. 7). In section (Text-fig. 1) the beginning of differentiation of the integument into layers (*i-1*, *i-2*, *i-3*) is visible. Next to the nucellus (*n*), evidence of a dark, narrow, inner integumentary layer (*i-3*) is marked by the presence of tannin cells. Outside of this is a broad white region (*i-2*), not yet sharply delimited on the dorsal side of the ovule from the brown tissues of bract and fertile scale to which the ovule is adnate.

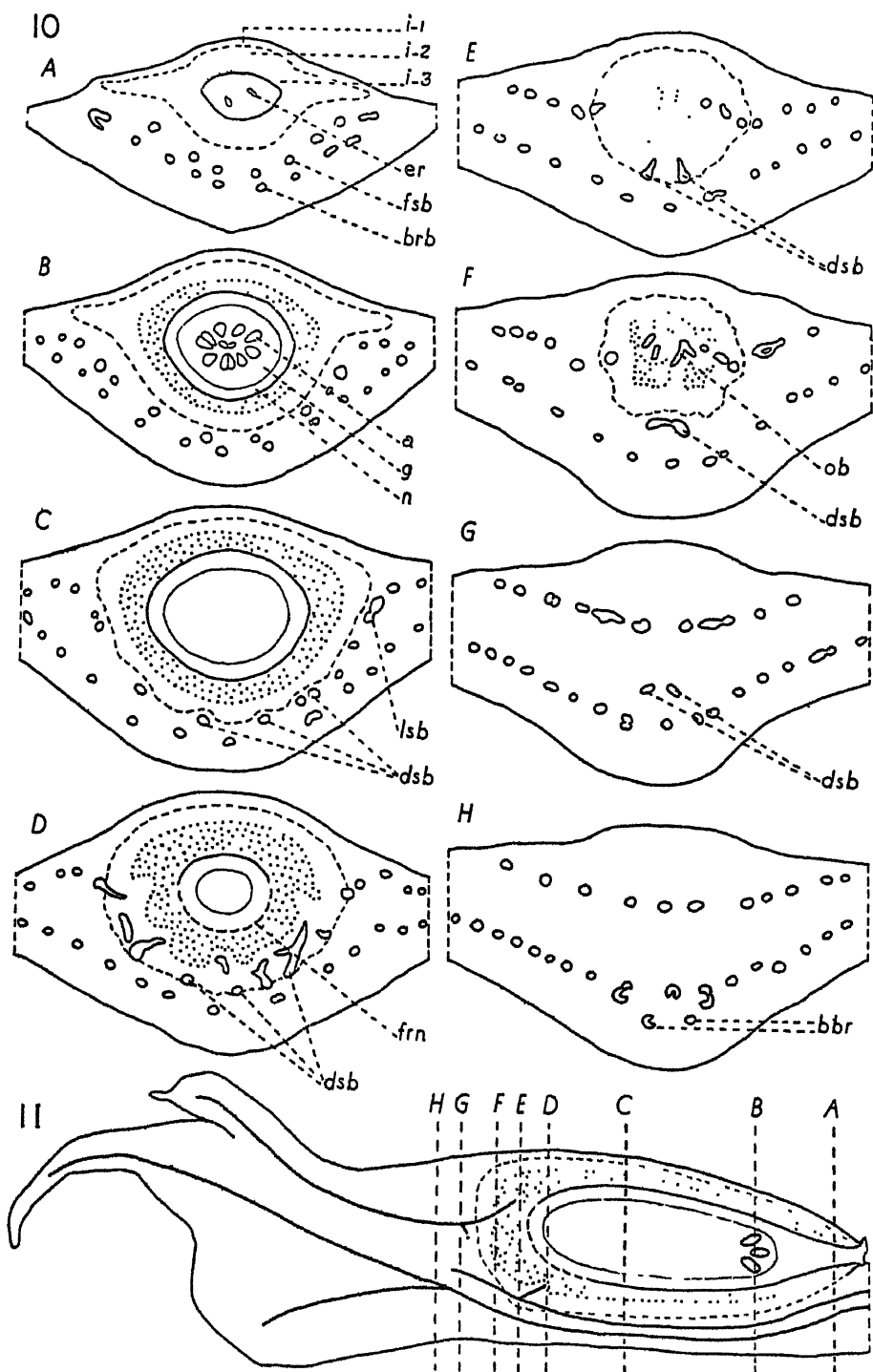
*A. Bidwilli* is remarkable as the only species, so far as is known, in which the vascular traces of the bract and fertile scale are unfused throughout and derived separately from the axis of the cone, the bract supplied by one trace, the scale by two (Worsdell, 1899; Eames, 1913). The lower series of bundles, derived from the single trace and supplying the bract, are normally oriented, the upper bundles, derived from the double trace and supplying the fertile scale, have been shown to be an inverted series. At this stage (Text-fig. 1), shortly after pollination, the fertile-scale bundles (*fsb*) under the ovule (in longitudinal section) appear to lie within the white area; the bract bundles (*brb*) lie at the edge of it, very close to the scale series. Fertile-scale bundles extend well into the free portion of the scale in *A. Bidwilli* in contrast with other species in which the fertile scale is less prominent and the free portion is completely or only partially vasculate. The ovular supply at this stage is not sufficiently differentiated to determine accurately.

At fertilization, a year later, in cones from Fresno, California, great enlargement has taken place (Text-fig. 11). The fertile scale is now nearly as long as the bract, the proportionate length of the fused region has increased, and the scale is now free only at the tip. The ovule at this stage is more than half as long as the cone scale.

In transverse and longitudinal sections the layering of the integument is clearly marked (Text-figs. 10, 11). On the ventral surface it has become differentiated into three layers: an outer, thin, dark-coloured tanniferous layer (*i-1*); a median, dense, white layer (*i-2*); and an inner, dark, tanniferous layer (*i-3*). The outer layer at maturity becomes the thin, papery layer (separable from the inner layers) which has already been described as continuous laterally with the scale and as holding the mature 'seed' on the scale unit (Pl. VI, Fig. 12; Text-figs. 15, 16, *i-1*). The median, white layer, which completely surrounds the ovule (*i-2*), becomes the hard, light-coloured, stony coat of the free 'seed' (Pl. VI, Figs. 5, 6). The inner tanniferous layer becomes

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TEXT-FIGS. 10-11. *A. Bidwilli*. Fig. 10: A-H. Series of diagrammatic transverse sections of bract-scale unit and ovule at fertilization, cut as shown in Fig. 11, showing vasculature of bract (*brb*), scale (*fsb*), and ovule (*ob*); layers of integument (*i-1*, *i-2*, *i-3*); gametophyte (*g*) and archegonia (*a*); erosion by pollen tubes (*er*); and branches of bract bundles (*bbr*) supplying apophysis. Further explanation in text.



FIGS. 10-11.

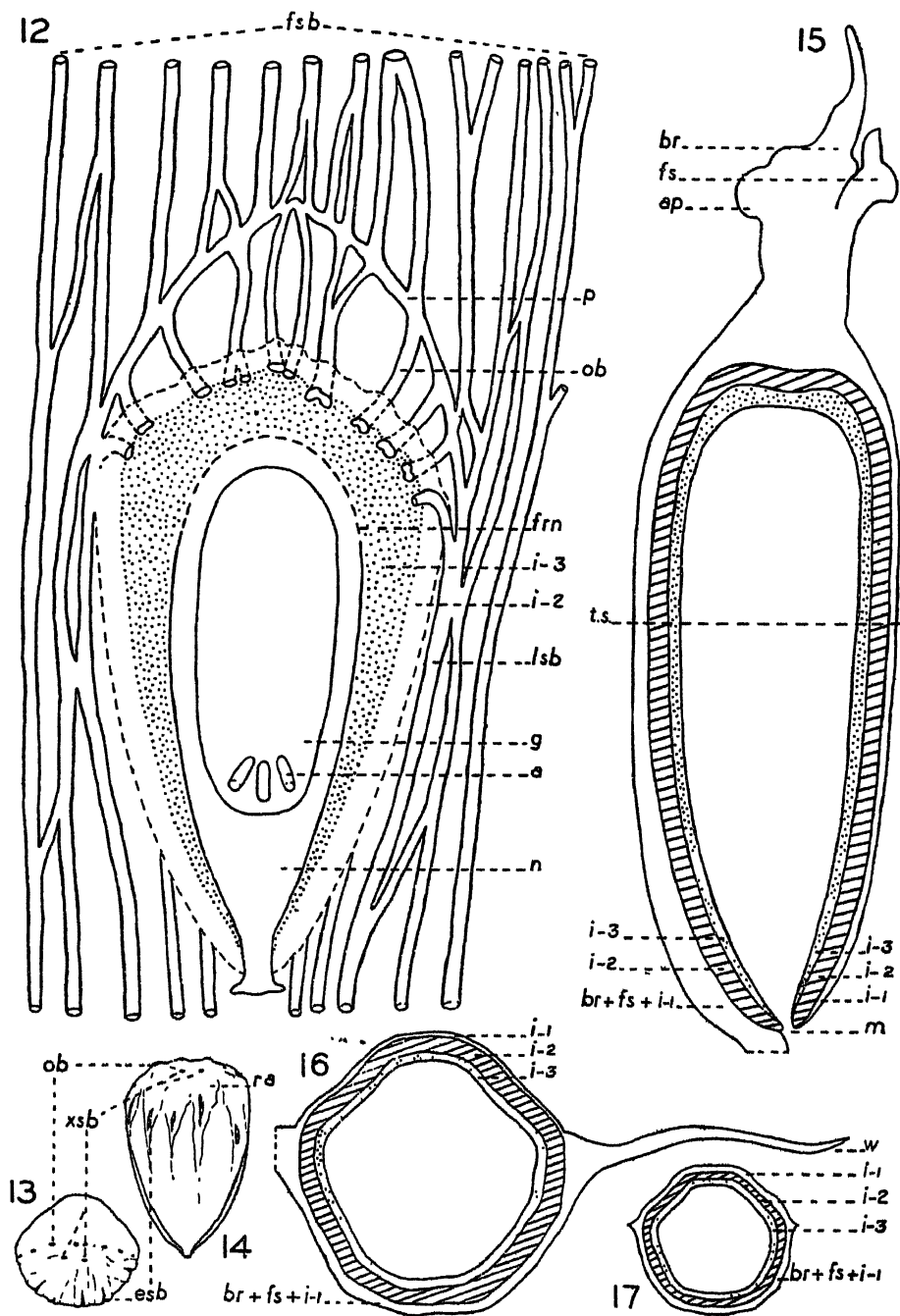
in the mature 'seed' a thin, dark layer, very thin at the micropylar end, considerably thicker at the chalazal end (Text-figs. 15, 16, *i-3*). Here it is fused with the nucellus (Text-fig. 12, *frn*), which is papery at maturity. Except for this fused region, cap-like in its extent, the nucellus is free from the integument.

On the under side of the ovule at the fertilization stage three distinct layers are distinguishable only at the micropyle, where the ovule is free from the fertile scale for approximately 1 mm. Otherwise, along the entire dorsal side of the ovule only two layers are distinguishable, the inner dark layer and the white stony layer (Text-figs. 10, 11). As in the adnate, inverted ovules of other conifers, integument and fertile-scale tissues merge with no line of separation. The dense, white, stony layer on the under side includes not only integumentary layers but some tissue of the cone-scale. This is evident in that it projects between the scale-bundles and even includes them near the chalazal region (Text-fig. 10, C, D). The stony layer is, therefore, not a morphologically distinct layer: on the ventral side of the ovule it represents a median layer of the integument, on the dorsal side, outer layers of the integument plus the adjacent tissues, even frequently bundles, of the fertile scale. The stony 'seed' coat does not represent integument only, but is partly integument and partly cone scale. The 'seed' of this species, therefore, is not morphologically a seed because it lacks part of the seed-coat on the ventral side (the thin, papery outer layer) and includes part of the cone scale on its dorsal side.

Serial sections of the bract-scale unit at the fertilization stage show clearly the course of the scale-bundles and the ovular supply. Transverse sections (Text-fig. 10, A-H), about 1 mm. from the micropyle, show the normally oriented lower arc of bundles supplying the bract (*brb*), and close above them the inverted bundles of the fertile scale (*fsb*). At a level within the cortex of the cone axis Eames (1913, p. 25) has shown these two series of bundles as forming a complete circle. At this level, just distal to the micropyle, the two

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TEXT-FIGS. 12-17. Figs. 12-16. *A. Bidwilli*. Fig. 12. Diagrammatic median longitudinal section of ovule in plane of scale showing its vascular supply (*ob*) from plexus (*p*) of anastomosing lateral fertile-scale bundles (*lsb*). The ovule, at fertilization, shows integument differentiated into an outer layer fused with scale and, here not shown, a median white layer (*i-2*), and an inner tanniferous layer (*i-3*) which is fused with nucellus in the chalazal region (*frn*). Within integument is nucellus (*n*), which contains gametophyte (*g*) with archegonia (*a*). ( $\times 5$ .) Fig. 13. Diagrammatic dorsal view of 'seed' showing three series of holes associated with vascular bundles; *ob*, entrance of ovular bundles; *esb*, entrance of fertile-scale bundles; *xsb*, exit of some fertile-scale bundles. Other fertile-scale bundles terminate under raised areas (*ra*). ( $\times \frac{3}{2}$ .) Fig. 14. Chalazal view of seed showing two series of holes (*ob*) and (*xsb*). Fig. 15. Diagrammatic longitudinal section of mature 'seed' and bract-scale unit. Bract (*br*) shows woody apophysis (*ap*) and is free from fertile scale (*fs*) only at its apex. 'Seed' coat on ventral side is differentiated into a thin, brown, separable, papery layer (*i-1*), a median (outer, on free seed) hard, white, stony layer (*i-2*), and an inner, brown, fibrous layer (*i-3*). On dorsal side, *i-1* merges with fused scale and bract. ( $\times 1\frac{1}{2}$ .) Fig. 16. Diagrammatic transverse section of mature seed and bract-scale unit showing layers of seed-coat (*i-1*, *i-2*, *i-3*) and lateral woody wings (*w*) of bract. ( $\times 1\frac{1}{2}$ .) Fig. 17. *A. angustifolia*. Diagrammatic transverse section of mature seed and bract-scale unit showing layers of 'seed' coat and lack of wings on bract. For comparison with Fig. 16. ( $\times 1\frac{1}{2}$ .)



FIGS. 12-17.

series are still frequently laterally connected by the peculiarly oriented end-bundles as shown in Text-fig. 10, A. The scale-bundles, however, at this level are pushed downwards, by the developing ovule, into an arc which lies close to that of the bract-bundles. Successive sections approaching the middle of the ovule show the scale-bundles lying increasingly closer to the stony white layer. This layer, even at the fertilization stage, shows the grooves in which the bundles lie which are so conspicuous on the stony coat of many of the 'seeds'. As the bundles approach the chalazal end they behave variously, depending upon position with relation to the ovule (Text-fig. 12). Strong lateral bundles (*lsb*) of the arc, lying close against, sometimes within, the stony white layer, approach one another just beyond the chalazal region. Here, an anastomosing plexus (*p*) is formed, from which branches (*ob*) bend downwards into the integument at the chalaza. These branches penetrate to the dark innermost layer of the integument (*i-3*). Their point of entrance is clearly marked on mature 'seeds' by a conspicuous row of holes at the larger end of the seed (Pl. VI, Figs. 1, 2, 4, and 5, *obe*). The original scale-bundles, and others from the plexus (Text-fig. 12, *fsb*), continue into the free portion of the fertile scale. Branches from these lateral bundles therefore furnish the strongest ovular supply.

Those bundles of the arc lying dorsal to the ovule often appear to be weaker bundles (Text-fig. 10, C-G, *dsb*). Their number, behaviour, and degree of prominence are variable. As they approach the chalazal end of the ovule, often lying in grooves of the stony layer, they send branches into the integument or before branching they may enter the stony layer (Text-fig. 10, C, D, E). In specimens from cultivated trees grown in California, the holes on mature 'seeds' marking the penetration of these dorsal scale bundles into the stony layer are near the middle of the 'seed' (Pl. VI, Figs. 2, 4, 5, and 11, *sbe*). Holes near the chalazal region marking their exit (Pl. VI, Figs. 2, 4, 5, *sbsx*) on the mature 'seed' are sometimes present, but are often inconspicuous or lacking. After giving off ovular branches some of these bundles appear smaller and weaker and continue only a short distance into the scale beyond the ovule (Text-fig. 10, G, H). Other dorsal scale-bundles seem to terminate in the stony layer, often under raised areas (Pl. VI, Figs. 8, 9, *ra*). In much larger, plumper 'seeds' from native trees in Australia, the dorsal scale-bundles themselves do not appear to enter the stony layer but branches from these bundles were found to enter nearly at the chalazal region. Worsdell (1899, p. 533) in his material of *A. Bidwilli* apparently did not find dorsal scale-bundles entering the ovule.

The lower, normally oriented bundles of the bract often lie very close to the scale-bundles and to the ovule. About the middle of the bract, a little beyond the 'seed', strong bundles branch downwards into the thick woody apophysis (Text-fig. 10, H, 11). In some bracts, in fact, these strong bundles can be followed from the original branching of the bract-trace directly to the apophysis, the weaker bundles continuing into the long leafy point of the bract as branches of the strong bundles. In other bracts all the bundles seem to be of the same size and importance.

# POLLEN TUBES AND NUCELLUS

A study of pollen tubes and nucellus in this species of *Araucaria* became possible in the spring of 1946. Dr. Hadsall kindly made weekly collections of cones during and for some weeks following pollination from trees in Fresno, California, and forwarded them immediately by air mail. Relatively fresh material could thereby be examined.

*Araucaria*, *Agathis*, *Saxegothaea*, and probably most species of *Tsuga* are unique among conifers in that pollen does not germinate on the nucellus but on the fertile scale, or on the bract, over which pollen tubes grow towards the micropyle. Doyle and O'Leary (1935 *a* and *b*) have described this condition in *Saxegothaea* and in *Tsuga*. In *Tsuga pattoniana* (Doyle and Kane, 1943), pollen germinates more often on a stigmatic flap of the integument than on the scale. Thomson (1905, 1907) first found the germinating pollen grains on the fertile scale of *Araucaria* and termed it the 'protosiphonogamic' method of fertilization; later Eames (1913) found numerous pollen tubes growing not only in the axil of the scale in *Agathis* but invading tissues of scale and cone axis. At the time of pollen germination in *Agathis* no micropyle is differentiated. In *Araucaria angustifolia* (*A. brasiliensis* aut.), moreover, as described by Burlingame (1913, p. 107), not even a trace of ovules can be found on the cone-scales at the time of pollination, usually late in April, in trees planted in California. The nucellus is usually not ready to receive the growing pollen-tubes until September or October.

A quite different condition was observed in *Araucaria Bidwilli*. At the time pollen is being shed the ovule is clearly visible. In 1946 the shedding of pollen from trees in Fresno, California, was apparently late, not occurring until the first week in May. In 1943 pollen was shed during the first week in April. In collections from both years, however, the nucellus and micropyle are well differentiated at this time, although the scales and ovules of April 1943 are slightly smaller than those of the 1946 collections. Another collection of young cones from native trees in Queensland, Australia, taken shortly before pollination, shows the ovule clearly differentiated, with micropyle and protruding nucellus (Text-fig. 2). These scales and ovules are still smaller than those of the April 1943 collection from Fresno and the nucellus does not protrude as much through the micropyle. In all collections the ovule forms only a slight elevation above the surface of the scale; the micropyle is round and slightly asymmetrical, in that it is deeper on the under side next to the scale (Text-figs. 5, 6). Through it the nucellus protrudes, its blunt end pushed against the axis of the cone. The upper margin of this blunt end, slightly flaring, and minutely and irregularly toothed, is thin and turned upward (Text-fig. 6).

No pollen grains unfortunately were found germinating. A few pollen tubes, however, which had already entered the nucellus, were first noticed on the scales of cones collected May 23, 1946, from 2 to 3 weeks after pollination. In a May 30 collection more numerous pollen tubes were found on the surface

of the scales and penetrating the nucellus. The ovule had increased in size from approximately 1 mm. in length at pollination to  $3\frac{1}{2}$  mm., and the nucellar flare was more pronounced (Text-fig. 9). The pollen tubes on the surface of the fertile scale were found to lie in grooves, as described by Thomson (1905); these grooves, however, somewhat broader than the pollen tube, do not appear to be furrows natural to the scales. Frequently the grooves run obliquely across the scale nearly at right angles to the natural slight irregularities. They run usually directly to the micropyle and frequently a small niche is present in the edge of the integument where a tube has passed into the micropyle towards a nucellus which is not protruding. Whether the grooves are formed by mechanical pressure or by erosion is not clear. A slight yellowing of the tissues where two grooves have run together on one scale suggests that actual erosion may have occurred. Some of the pollen tubes run towards the cone axis, where they turn and often enter the nucellus from the side. The pollen tubes are thin, translucent, and delicate at this time (Text-fig. 9, *pt*), in contrast to their appearance a year later at fertilization, when they have increased greatly in diameter and are prominent and numerous at the tip of the nucellus (Text-fig. 4, *pt*). Their course in the nucellus appears to be devious with loops and turns. Some tubes, after entering the nucellus, apparently emerge and enter again, forming a loop at the surface (Text-fig. 4). Most of the ovules from collections taken at the fertilization stage show numerous pollen tubes and the nucellar flare is more 'fringed', apparently from erosion by the tubes.

Although in some ovules the nucellus is still protruding at this time, in most the integument at the micropyle completely hides the nucellus, apparently as a result of growth following pollination.

#### DISCUSSION

Since the recent publication of three more parts of Florin's remarkable monograph on the fossil conifers it is now possible to come to a final satisfactory interpretation of the long-disputed nature of the araucarian cone-scale unit. And since the cone-scale of *A. Bidwilli* apparently represents the most primitive condition among the living araucarians, it reveals most clearly in its structure the connecting links of the living group with still more primitive ancestors.

The types of vasculature in the cone-scale unit of species of *Araucaria* fall naturally into three groups (Eames, 1913). In *A. Bidwilli*, the only representative of group I, so far as is known, the vascular supplies of bract and fertile scale are derived separately from the cone axis, that of the fertile scale as two traces, a condition characteristic of axillary shoots. In species of the *Eutacta* section, group II (with the possible exception of the New Guinea species which have not been investigated), the bract and fertile scale supplies are united at their source, but separate immediately, either in the cortex of the cone axis or in the base of the cone-scale unit, into independent series supplying bract and fertile scale. In group III, characteristic of the two

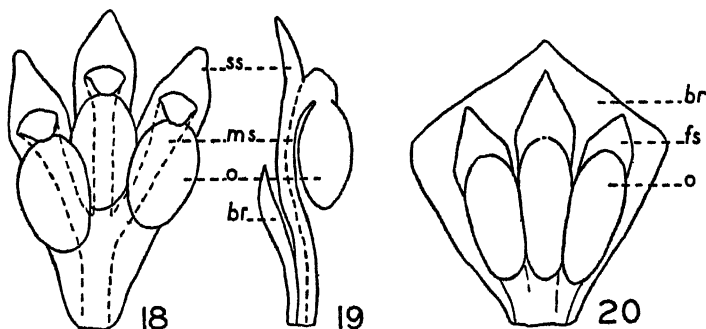
South American species of *Columbea*, the bract and fertile-scale series is united from its origin throughout its whole extent.

The independent origin from the cone axis of two vascular supplies, respectively, for bract and fertile scale in *A. Bidwilli* indicates the fundamental axillary nature of a fertile scale now more or less intimately fused with the bract for a considerable part of its length. The primitive axillary structure in Paleozoic conifer cones from which the modern coniferous fertile scale has been derived is shown by Florin (1944, p. 477) to have been a short shoot bearing spirally arranged sterile and fertile scales. Ovules were borne terminally on some of the scales or megasporophylls. During the subsequent process of reduction, flattening, and fusion among the members of this shoot, the ovule-bearing structures or megasporophylls often each became intimately associated, by fusion, with a sterile scale. The ovule, thereby, appears to be borne on the surface of the scale. But on fossil cone-scales from the early Mesozoic (Florin, 1944, p. 496), evidence is still visible that the ovule was terminal on a proximal megasporophyll which had become fused with a distal sterile scale. Such 'seed-scale complexes' (as Florin terms them) may be seen in the Mesozoic *Schizolepis*, cited by Florin (1944, pp. 484-96), as a prototype of the modern greatly reduced araucarian cone-scale. In *Schizolepis* the cone-scale was made up of a greatly reduced shoot bearing three flattened, distal sterile scales laterally fused to one another at the base for about half their length (Text-fig. 18), their distal portions free. On the surface of each sterile scale was a longitudinal ridge, the ridges becoming confluent at the base. These ridges represent three proximal megasporophylls (*ms*) fused throughout nearly their entire length each with a sterile scale (*ss*), and at the base laterally with each other. At their apices the megasporophylls were recurved and bore each a free pendent ovule (*o*) (Text-figs. 18, 19). The whole complex was free from a subtending bract (*br*).

Evidence exists in the vascular anatomy of the cone-scale unit of *A. Bidwilli* that the single ovule represents a surviving one of three (Text-fig. 20). It is significant that the vascular bundles supplying the ovule are mainly derived from strong scale bundles of the inverted series lying lateral to the ovule. The weaker median bundles of the inverted series dorsal to the ovule frequently die out. It seems logical to suppose that these three groups, the two strong lateral and the weaker median, may each have supplied an ovule. Since the median group of bundles is the weakest and does not contribute an ovular supply to any extent, it seems probable that the median ovule has been lost and that the remaining ovule represents a surviving lateral one. The free portion of the fertile scale distal to the ovule, more prominent in *A. Bidwilli* than in other species, is supplied from the plexus, formed by anastomoses between these lateral bundles, and represents possibly two fused lateral sterile scales, the median one having been crowded out. To these the megasporophylls have likewise (phylogenetically) long been fused.

Although the vascular anatomy of the cone-scales of nearly all except the three New Guinea species has been studied, little careful attention has been

given to the origin of ovular bundles and to the behaviour of the bundles in the critical chalazal region. It is evident, however, from published figures that ovular supply is similarly derived from lateral bundles in *A. araucana* (*A. imbricata*) (Seward and Ford, 1906, Fig. 27) and *A. angustifolia* (*A. brasiliensis*) (Van Tieghem, 1869, Pl. 15, Figs. 63–73), even though the bract and scale series are here fused throughout their length and no scale bundles go far beyond the chalazal region. Eames (1913, p. 26) described the ovular supply in *A. excelsa* as being derived from lateral bundles of the 'lower series',



TEXT-FIGS. 18–20. Figs. 18–19. *Schizolepis*. Fig. 18. Diagrammatic reconstruction of bract-scale unit of *Schizolepis*, based on photograph of fossil cone-scale by Florin (1944, Taf. clxxxii, Abb. 20, 21). *ss*, sterile scale; *ms*, megasporophyll; *o*, ovule; *br*, bract. Fig. 19. Diagrammatic reconstruction of radial section (in vertical plane) of bract-scale unit of *Schizolepis* shown in Fig. 18. Fig. 20. Diagrammatic reconstruction of possible ancestral type of araucarian bract-scale unit. Further explanations in text.

while the few small inverted bundles dorsal to the ovule die out. He also described strong lateral bundles passing out into the scale in *A. columnaris* (*A. Cookii*). From Strasburger's (1872, Taf. VII, Figs. 63–71) figures of the cone-scales of *A. Cunninghamii* also, the ovule supply appears to be derived from lateral bundles of the series. Although Aase (1915) studied the cone scales of several araucarian species the origin of the ovular supply in these is not clear.

Mitra (1927) also has found strong evidence in other species that the single ovule has been reduced from two or three and that the distally free scale represents a fusion of two. In *A. montana* and *A. Rulei* in a region at the base of the cones transitional between the sterile and fertile scales he found cone-scale units with two free fertile-scale tips and two aborted ovules. Higher in the cone these scales tended gradually to fuse into one. Such scales were found also by the present authors in a cone of *A. Bidwilli*. Mitra found a still more significant condition in sectioning scales, both fertile and sterile, from this transitional region. Often in scales of *A. Rulei*, *A. montana*, *A. araucana* (*A. imbricata*), and *A. Bidwilli* he found the bundles of the inverted series segregated into two or three groups. In scales with two free tips and two ovules the lateral ones of the three groups corresponded with the laterally

placed ovules. The median group was often weak and represented by few bundles.

Both Mitra and Florin have concluded that the one-seed condition in *Araucaria* is derived, and Florin has further presented in *Schizolepis* a cone scale from which that of *Araucaria* may be derived. Although these authors believe that the median ovule has been the one retained, vascular evidence in *A. Bidwilli* and other species suggests that it is a lateral one. The frequently weak development of median bundles dorsal to the ovule suggests that the middle seed-scale was the first to be lost and that the remaining lateral ones thereafter became fused.

#### SUMMARY

The morphology of the ovule, 'seed', and cone scale of *A. Bidwilli* has been investigated and certain peculiarities noted. The cone-scale unit is composed of a large, heavy, woody-winged bract and prominent fertile scale fused with the bract for two-thirds of its length. The 'seed', with its light-coloured stony coat, is shed from the bract-scale unit in *A. Bidwilli*, unlike the seeds in all other species of the genus which are retained on the fertile scale, forming nut-like or samara-like bract-scale units. The free 'seed' in this species is morphologically not a seed because it lacks the outer layer of the integument on its ventral side and includes some tissues of the fertile scale on its dorsal side. At no time in ontogeny is the ovule or seed 'enclosed' by tissues of the fertile scale. The vascular supply of the bract-scale unit and ovule have been studied in detail and observations made on the course of the pollen tubes. Evidence from vascular anatomy supports the view that the single ovule in *Araucaria* is a survivor of three. The axillary nature of the araucarian fertile scale is reaffirmed and Florin's interpretation of modern coniferous cone scales as based on extensive fossil evidence is cited.

This paper presents, in part, morphological evidence as a basis for a modification of the sectional treatment of the genus to be proposed in a succeeding paper.

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## DESCRIPTION OF PLATE

Illustrating Assistant Professor M. H. Wilde and Professor A. J. Eames's article on 'The Ovule and "Seed" of *Araucaria Bidwilli*. I. Morphology.'

Figs. 1-12. *A. Bidwilli*.

Fig. 1. Chalazal view of 'seed' showing holes (*obe*) marking entrance of ovular bundles. ( $\times 1$ .)

Fig. 2. Dorsal view of 'seed' showing holes (*sbe*) marking entrance of some fertile-scale bundles (or their branches); one hole (*sbx*) marking exit of one fertile-scale bundle; other holes (*obe*) marking entrance of ovular bundles. ( $\times 1\frac{1}{2}$ .)

Fig. 3. Micropylar view of 'seed', showing remnants (a thin layer) of fertile-scale tissue adnate to dorsal side of 'seed' with vascular bundles lying in grooves. ( $\times 1\frac{1}{2}$ .)

Figs. 4, 5. Lateral and dorsal views of 'seed' showing holes (*sbe*) marking entrance of some fertile-scale bundles (or their branches); other holes (*sbx*) marking exit of some fertile-scale bundles; and terminal 'humps' (*obe*) in chalazal region marking entrance of ovular bundles. ( $\times 1$ .)

Fig. 6. Ventral view of 'seed' showing light-coloured stony layer of 'seed' coat. ( $\times 1$ .)

Fig. 7. Dorsal view of same 'seed' as in Fig. 6, showing remnants of fertile-scale tissue adnate to stony layer of 'seed' coat with scale bundles lying in grooves. ( $\times 1$ .)

Figs. 8-11. Dorsal views of 'seeds' showing fertile-scale bundles (*sb*) entering 'seed' coat at *sbe* and lying under light-coloured raised areas in coat at *ra*. Lateral ridges on 'seed', (*r*).

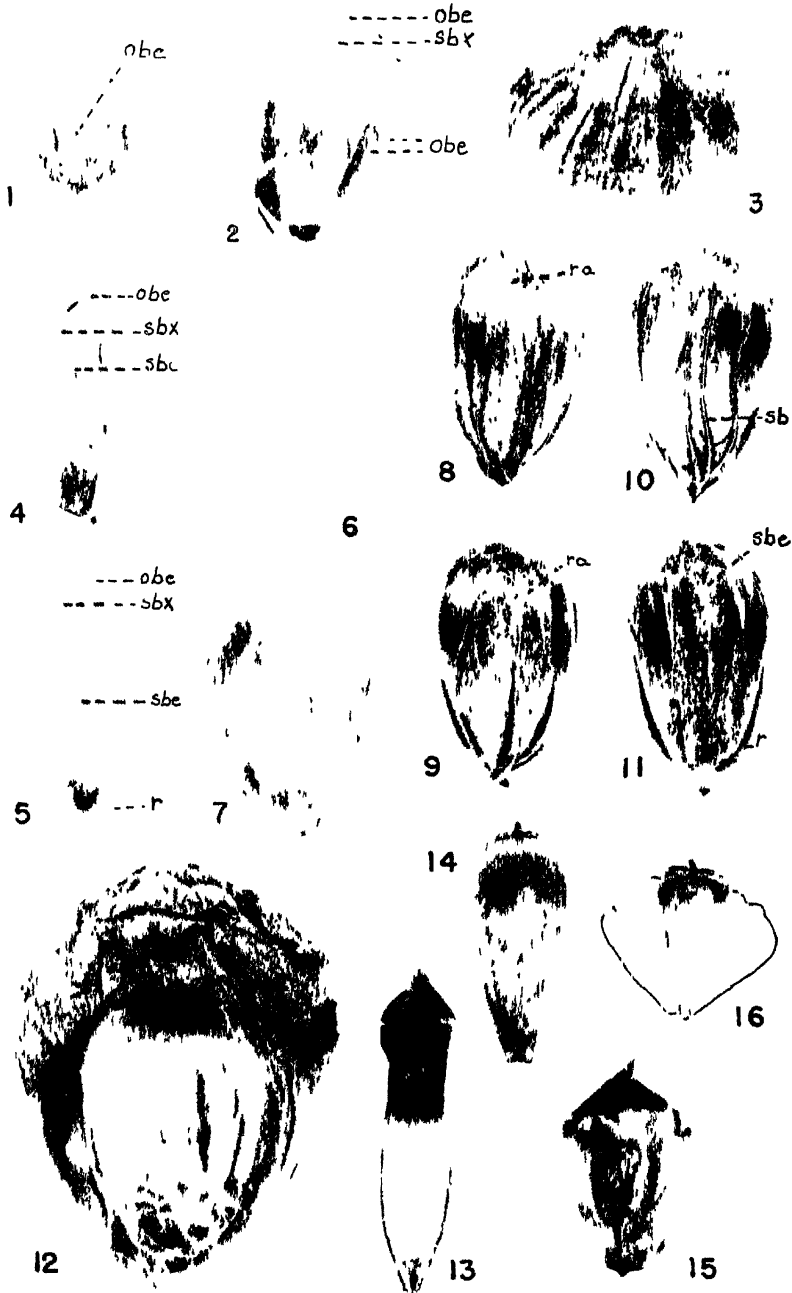
Fig. 12. Bract-scale unit with woody wings and ripe 'seed' showing separable, dark, papery, outer layer of 'seed' coat torn in several places where light-coloured, stony layer of 'seed' is visible. ( $\times 1$ .)

Fig. 13. *A. araucana*. Wingless nut type of mature bract-scale unit and seed. ( $\times 1$ .)

Fig. 14. *A. angustifolia*. As above. ( $\times 1$ .)

Fig. 15. *A. columnaris*. Samara type of mature bract-scale unit and 'seed' with thin wings. ( $\times 1$ .)

Fig. 16. *A. Cunninghamii*. Samara type of mature bract-scale unit and 'seed' with very thin, papery wings. ( $\times 1$ .)





# A Preliminary Anatomical Note on Vascular Wilt Disease of the Oil Palm (*Elaeis guineensis*)

BY

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With Plate VII

## INTRODUCTION

IN 1946 Professor C. W. Wardlaw described a new vascular wilt disease of oil palms in the Belgian Congo. Affected palms showed external symptoms of wilting, fungal hyphae were observed in the necrosed wood vessels, and a species of *Fusarium*, identified by Dr. W. Gordon as a strain of *Fusarium oxysporum*, was isolated from affected palms in different regions of the Belgian Congo (Wardlaw, 1946, 1946a). The appearance and morphology of the fungus on standard media have been described by Gogoi (1948).

A considerable time must necessarily elapse before inoculation experiments can be brought to completion. In the meantime progress can be made in investigating this new disease (i) by observation of its symptoms in different environments and in palms of different age, (ii) by studying the organisms isolated from necrosed vessels, and (iii) by observing the distribution of the pathogen in the tissues and the consistency with which it can be demonstrated in the vessels of wilted palms. The present paper deals with the third of these aspects, the writer having worked systematically through the materials collected and preserved by Professor Wardlaw.

## MATERIALS AND METHODS

The materials available for study consisted of tissues which had been taken from the lower part of infected trunks. These had been preserved in 70 per cent. alcohol. Materials collected at Yangambi, Elizabetha, Leverville, and Brabanta all showed the discoloured vascular bundles typical of the disease. Sections of necrotic vascular tissue taken from near the apex of the trunk of oil palms have recently been received from Messrs. S. de Blank and F. Ferguson in Nigeria.

Hand sections were mounted in glycerine jelly containing either safranin or gentian violet. Permanent preparations using the same stains were also made.

## OBSERVATIONS

All the materials examined showed fungal filaments in the wood vessels, some materials being more highly infected than others (Pl. VII, Fig. 1). The

mycelium was typically found in the wide pitted vessels of the vascular bundles, but was also observed in some of the narrower vessels. The fungus was not seen outside the vascular bundles.

The hyphae were septate, branched, and usually followed the longitudinal axis of the vessel. The diameter of the filaments varied from  $1.5\mu$  to  $3\mu$  in the Congo material and from  $2\mu$  to  $5\mu$  in the Nigerian. Conidia which were found resembled the microconidia of *Fusarium*, but the observations made were not such as to enable the fungus to be identified. In both the Congo and the Nigerian material chlamydospores were observed in the vessels (Pl. VII, Fig. 2).

The yellowish-brown discoloration of the vessels is associated with the presence of a gum within some of the vessels. Some vessels are completely blocked by gum and frequently, but by no means generally, show fungal filaments embedded in the gum (Pl. VII, Fig. 5). In many sections no fungus was found in the discoloured vascular bundles. In some preparations tiny droplets of gum could be observed on the mycelium within the vessels, although gum was absent from other parts of the vessels.

Many large vessels were completely filled with tyloses (Pl. VII, Figs. 3 and 4). No mycelium was observed in these vessels, but gum was present between the tyloses. Until the vascular tissues of healthy oil palms can be more thoroughly investigated, it cannot be known to what extent the presence of tyloses is indicative of the presence of a pathogen. In general, the appearance of infected vessels, the distribution of the hyphae, the presence of gum, and the development of tyloses are closely comparable with the observations made by Wardlaw (1931) on the vascular wilt disease of bananas which is also caused by a strain of *Fusarium oxysporum*.

The Nigerian sections were from much younger tissues and did not show the presence of gum or tyloses. The vascular strands were not as darkly coloured; in fact they were originally of an orange colour.

#### SUMMARY

In specimens of vascular wilt disease of the oil palm collected in the Belgian Congo and in sections from wilted palms received from Nigeria, fungal hyphae were consistently observed within the vessels. Hyphae were not observed outside the vascular bundles.

No identification of the fungus within the tissues has been possible, but the presence of chlamydospores, and of conidia resembling the microconidia of *Fusarium oxysporum*, support the view that this fungus is the pathogen.

The presence of gum and tyloses within the necrosed vessels is recorded.

The writer wishes to express his thanks to Professor C. W. Wardlaw for providing the material and for his advice during the investigation, and to Mr. E. Ashby for the preparation of the photographs.

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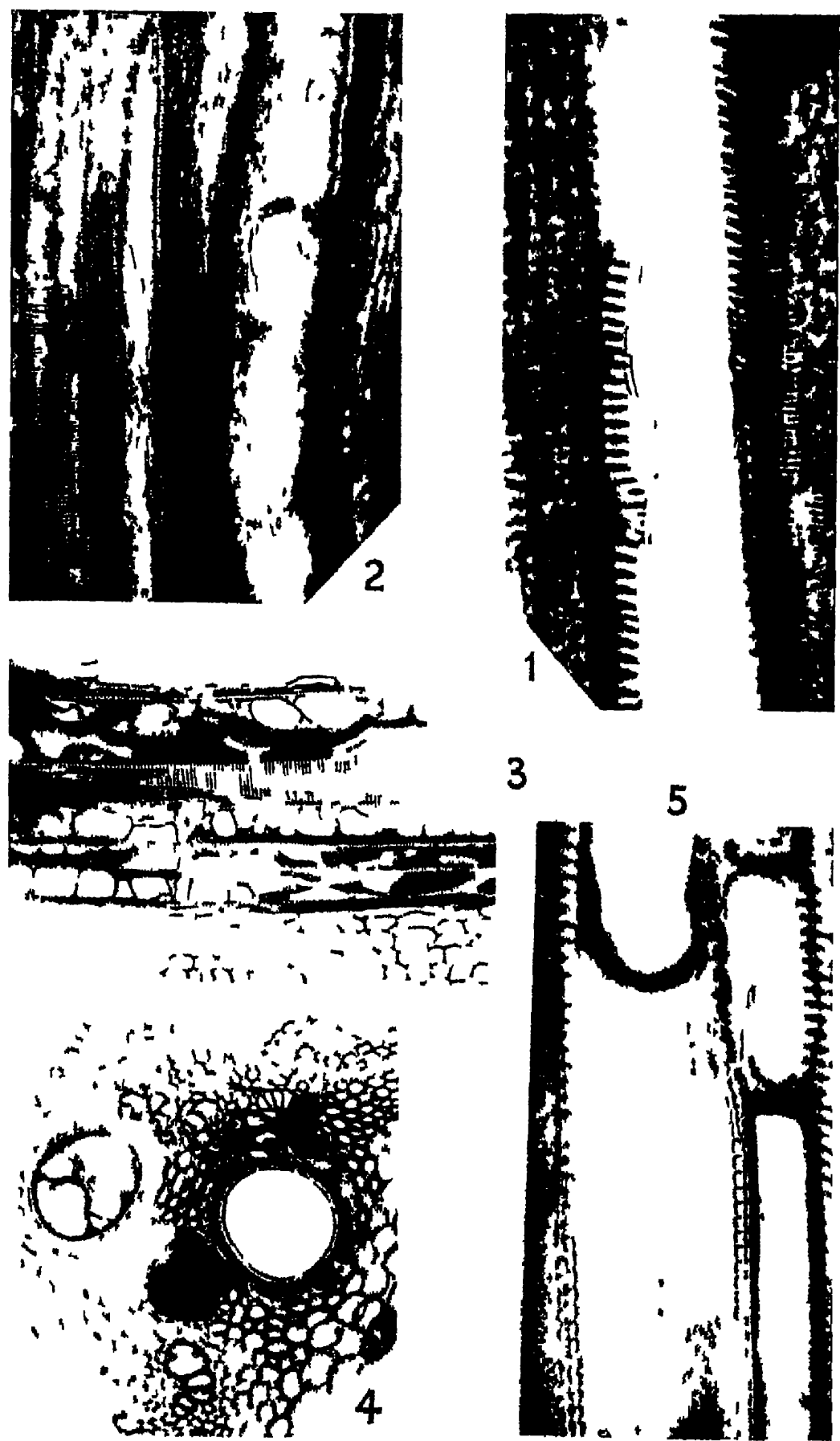
EXPLANATION OF PLATE

Illustrating Mr. W. G. Kovachich's paper on Vascular Wilt Disease of the Oil Palm

All figures are from untouched photographs.

- Fig. 1. L.S. of vascular bundle showing chlamydo-spores in hypha. ( $\times 200$ .)  
Fig. 2. L.S. of vascular bundle showing mycelium in the vessels. ( $\times 70$ .)  
Fig. 3. L.S. of vascular bundle showing the presence of gum and tyloses in the vessels.  
( $\times 70$ .)  
Fig. 4. T.S. of bundle showing tyloses and gum in the vessels. ( $\times 70$ .)  
Fig. 5. L.S. of vessel showing fungal hypha embedded in gum. ( $\times 360$ .)





KOVACHICHII—WILT DISEASE OF OIL PALM



## NOTE

**A simple torsion balance.**—During the course of physiological investigations the necessity frequently arises for the rapid weighing of a succession of small samples with a high degree of accuracy. With a precision balance of the usual chemical type speed and accuracy are mutually incompatible and, if the full sensitivity of the balance is to be utilized, an appreciable time is required for each individual weighing. The spring torsion balance, however, does not suffer from this disability, and balances have been constructed by Fabergé (*J. Sci. Instr.*, xv, 1938) with an absolute sensitivity as low as 0.001 mg. and which are practically dead-beat.

The present instrument was primarily designed for weighing leaf samples ranging in weight from about 10 to 20 mg., after drying, and has a maximum capacity of more than 150 mg. with a sensitivity better than 0.1 mg.

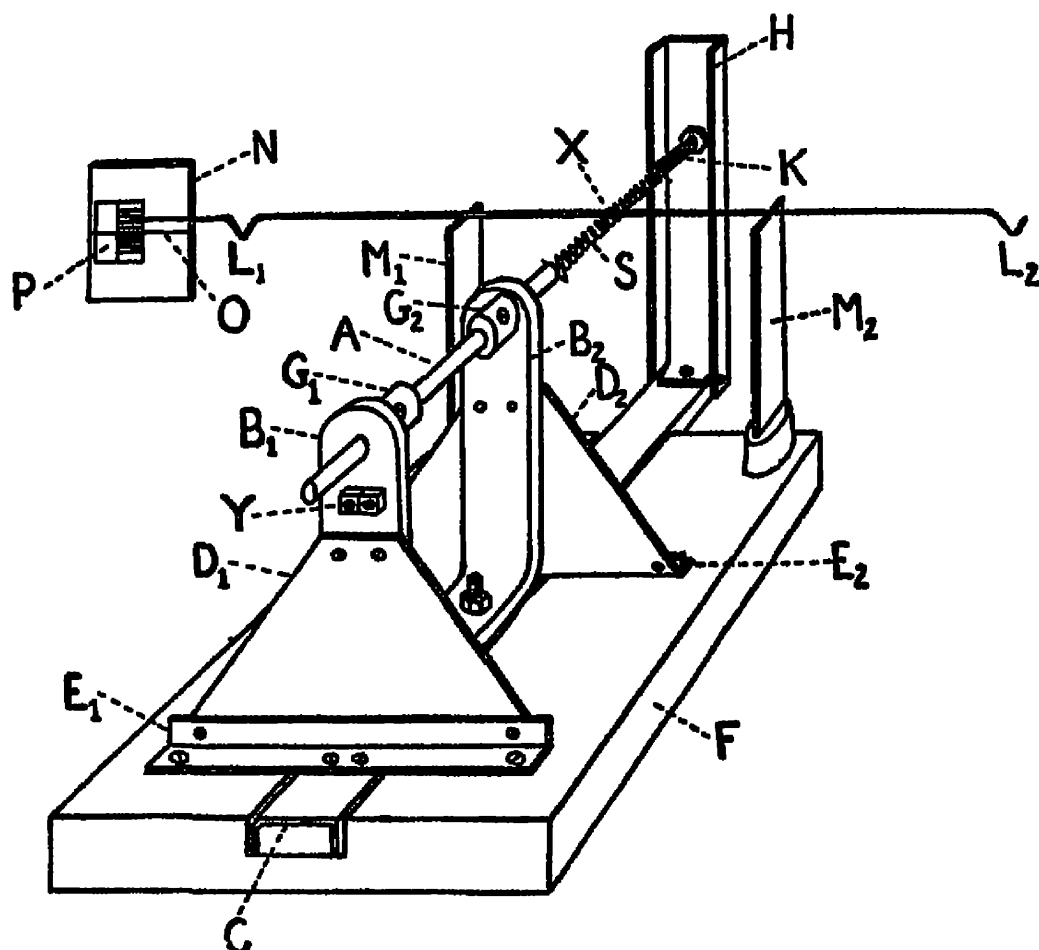
**Construction.** The principal parts of the balance are shown in the figure. The helical spring *s*, carrying the beam  $L_1L_2$ , is firmly fixed at its far end, while the near end is attached to one end of the rotating shaft *A*. A slow-motion drive is mounted on the near end of this shaft and a weighing operation merely consists of rotating this drive until the pointer of the balance is brought back to the null point, by means of the restoring torque applied to the near end of the spring.

The most difficult feature of the construction proved to be in securing the necessary degree of lateral rigidity in the shaft bearings  $B_1B_2$ . The bearing assembly consists of a very heavy U-shaped piece of brass, taken from an old gramophone motor, which was first bolted down on to a 12-in. length of  $\frac{7}{8}$  in. by  $\frac{1}{2}$  in. heavy-gauge brass channel *c*. Trapezoidal pieces of heavy brass sheet,  $D_1$  and  $D_2$ , were then cut out and firmly bolted to the uprights of the U as shown. The bottom edges of these pieces were next bolted to two cross-lengths of  $\frac{5}{8}$  in. brass angle  $E_1$  and  $E_2$ , which were themselves bolted firmly to the channel *c*. Unfortunately this arrangement failed to give sufficient rigidity due to an element of twist in the channel *c* upon which everything was mounted. Accordingly, in order to secure the requisite lateral rigidity, it was found necessary to screw the whole assembly firmly down on to the hardwood block *F*, which was channelled out as shown to fit *c*.

The shaft *A* is made from  $\frac{5}{8}$  in. brass rod, turned down to  $\frac{1}{4}$  in. at the near end to fit the drive, axial movement being prevented by the collars  $G_1$  and  $G_2$ , which are pushed up tight against the bearings before being locked on the shaft. The back attachment for the spring consists of another piece of brass channel *H*, mounted vertically on the end of *c*, and carrying a 6 B.A. bolt *K* in the same axial line as the shaft *A*. This bolt is secured by lock-nuts, back and front, enabling it to be turned in its seating when necessary to adjust the zero.

The spring itself consists of about 100 turns of 28 S.W.G. phosphor-bronze wire closely wound on an  $\frac{1}{8}$ -in. mandrel, the actual winding being done on a lathe with the aid of a spring-winding tool. It is mounted in the balance by bending the ends of the wire across the axis of the helix and soldering them into slots cut in the end of the shaft *A* and the bolt *K*. As mounted the spring is stretched to about twice its original length in order to separate the turns.

The beam  $L_1L_2$  is 40 cm. in length from hook to hook and is made by drawing out a piece of thin glass rod in such a way that it is thickest at the centre  $X$ , where it is attached to the middle turn of the spring  $S$  by a small blob of shellac, care being taken not to overheat the spring during this operation. The left-hand end of the beam beyond the hook  $L_1$  is drawn out into a fine pointer, which is blackened with



Projection drawing of balance with slow-motion drive and vernier removed.

indian ink. Observation of this pointer is facilitated by the use of a small square of mirror  $N$  on which a thin horizontal line  $O$  is scratched across the backing. A small piece of ivory scale  $P$  is glued to the face of the mirror in such a way that its centre line coincides with the line  $O$ . The beam is provided with two arrestments,  $M_1$  and  $M_2$ , which consist of ordinary microscope slides vertically mounted in slotted corks fixed to the block  $F$ . The heights of these slides are adjustable, enabling the free swing of the beam to be set to a convenient working value.

The Muirhead slow-motion drive used locks on to the end of the shaft  $A$  and is positioned by means of the keyway  $Y$ , fixed to the front of the bearing  $B_1$ . The drive has a 6-in. circular dial graduated into 300 divisions over half its circumference and is provided with a vernier reading to  $\frac{1}{10}$ th of a division. This is fixed in position at the top of the dial by means of a small strip of brass bolted behind the keyway block  $Y$ .

For use the balance is screwed down on to a wooden base-board measuring 20 in. by 14 in., on which an upright is erected to support the mirror  $N$ . Accurate observation of the zero position of the pointer is further assisted by the use of a lens mounted opposite near the front edge of the base-board. A wooden cover, open at the front,

and measuring 8 in. by 10 in. by 14 in., encloses the working parts, the ends  $L_1L_2$  of the beam emerging through slots cut in the sides. A small tinplate cover, sliding in guides fixed to the base-board, protects the end of the beam  $L_2$ , on which the object to be weighed is suspended, from air currents, while another three-sided tinplate protector is fixed in position above the exposed end of the beam  $L_1$ . A second lens and a 2.5-volt flash-lamp bulb are also fixed in suitable positions to facilitate reading the vernier scale of the main dial.

The total cost of constructing this instrument was about £4, of which amount £3. 3s. is accounted for by the slow-motion drive.

*Calibration.* The balance was calibrated by weighing various lengths of fine wire on it and then reweighing the lengths as accurately as possible on a high-grade chemical balance. Some representative readings are given below:

Dial reading.	Dial reading less zero value.	Weight, mg.	Weight in mg. per dial division.
2.6	0.0	0.00	—
56.4	53.8	38.73	0.720
92.0	89.4	64.46	0.721
126.6	124.0	89.18	0.719
155.7	153.1	110.08	0.719
220.2	217.6	156.42	0.719

The figures in the last column show that the scale is sensibly linear over the range tested, and, since readings can be repeated to 0.1 of a dial division using the vernier, that the absolute sensitivity is 0.07 mg.

Using this balance a complete weighing operation can be carried out in less than a minute.

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Preliminary Results obtained with an Apparatus for  
the Study of Salt Uptake and Root Respiration of  
Whole Plants

BY  
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With eleven Figures in the Text

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INTRODUCTION

SALT uptake by plants may be considered from two aspects: first, as part of the general process of growth, and second, that of the entry of ions and molecules into plant cells. The work here reported arose from previous

<sup>1</sup> This work was carried out while the first author held a Beit Research Fellowship at the Imperial College of Science and Technology; it incorporates materials submitted for a Ph.D. degree of the University of London.

investigations of nutrition in the barley plant. It is well known that the concentration of nutrient ions rises continuously during early stages of growth in this plant, and that this accumulation is one of the controlling factors in the early phase of rapid vegetative development. Indeed, the whole yielding capacity of the plant is determined during this stage, for the effects of early starvation cannot be eliminated by nutrient supply at a later stage after the ear primordia have been differentiated. This work was begun with the aim of studying the factors concerned in this phase of rising nutrient concentration within the plant.

So far as the analytical aspects of the problem are concerned much recent work has been done, notably by Hoagland (1944), Lundegardh (1945), Steward (1935, 1937), and their collaborators. It is now apparent that the metabolic processes in the cells of absorbing tissues are involved in the process of salt uptake. In view of the capacity of cells to accumulate salts against a concentration gradient the energetics of the process have assumed importance and have naturally led to a study of the relation between respiration and salt absorption. Steward has always insisted that accumulation against a concentration gradient can only occur in actively growing tissues, such as roots with active meristems or storage tissues capable of renewed meristematic activity. Hoagland has also stressed this view. A great deal of speculation on the underlying mechanism has appeared, and Lundegardh (1939, 1945) particularly has put forward schemata of great theoretical interest.

It is not intended in this paper to enter the controversial field. The aim of this work is to produce a body of data based upon a factorially designed experiment in which the interaction of the factors of concentration and oxygen supply is explored over a wide field. The concomitant variation in respiration and salt uptake with varying combinations of the factors has been studied so that a comprehensive body of data may be presented and examined in the light of some current theories. The whole plant was used in these experiments, and efforts were made to standardize the material as much as possible by controlling the conditions of growth, but to overcome the difficulty introduced by expansion of the root system during the experiment it was held to be desirable to reduce the period of the experiment to the smallest possible duration. In previous work only Lundegardh and Burström (1935) have followed the same procedure of using whole plants, and in some respects the apparatus designed for this work resembles that used by them. It has been possible, however, to devise a closed, circulating system, kept sterile, from which carbon dioxide of respiration was continuously removed and absorbed.

#### APPARATUS AND METHODS

##### *Apparatus*

A diagram of a single unit of the apparatus is shown in Fig. 1. It consisted of a root chamber with subsidiary devices to circulate the nutrient solution round the roots, withdraw and collect the respired carbon dioxide, and aerate

the solution with any desired concentration of oxygen, while maintaining the roots at atmospheric pressure.

The root chamber was funnel-shaped and had a volume of approximately 100 ml. It was made of glass and painted black on the outside. Into the

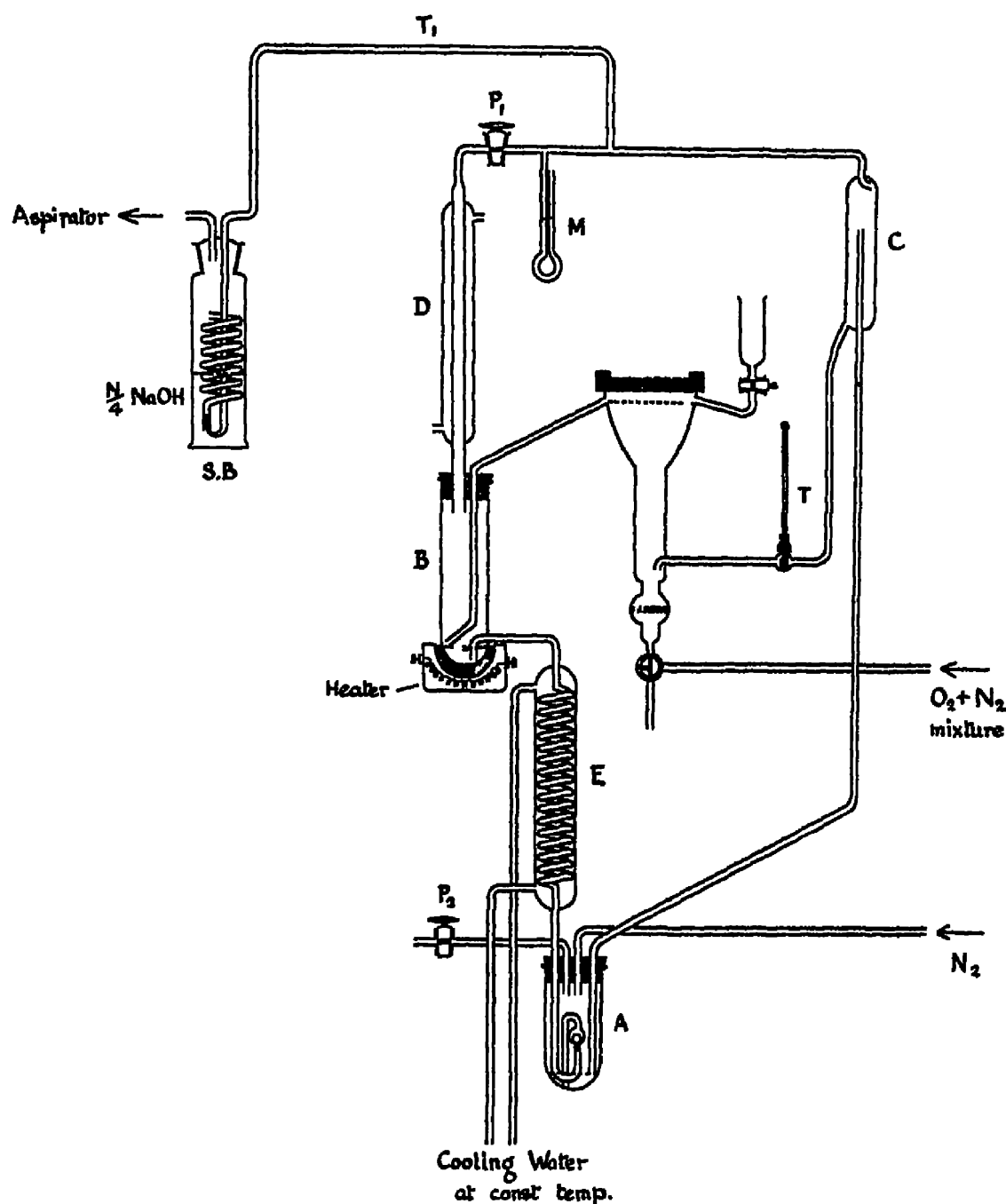


FIG. 1. Diagram of a single unit of the apparatus. For details see text.

lower portion of the funnel a sintered glass disk was fused which broke the aerating gas into fine bubbles. Three side-arms were attached (Fig. 1), one for filling the chamber and the other two for circulating the culture solution through the chamber. The upper rim of the funnel was ground flat and engaged with a circular groove on the lower surface of a bakelite disk which formed the lid of the funnel and held the plants which were to be studied. The joint between the rim of the funnel and the bakelite disk was made airtight with a melted mixture of sodium cerate and bees-wax.

The culture solution was caused to circulate in the apparatus by purified nitrogen under pressure. This gas was admitted into vessel A and forced any liquid present, interspersed with gas-bubbles, up the glass tube to the separating bulb C, where the gas escaped and the liquid fell back into the root chamber. Any excess liquid in the root chamber was blown by the aerating gas into the boiling-tube B. Here the liquid was boiled continuously by means of an external electrical heater and carbon dioxide and other gases driven off through the condenser D. Excess liquid in the boiling-tube drained through another condenser E and a glass poppet-valve, back into vessel A. The poppet-valve prevented any back-flow of gas or liquid up the condenser, and the rate of flow of liquid round the apparatus was controlled by a needle-valve, which regulated the flow of the nitrogen gas into vessel A.

The culture solution was aerated by a mixture of oxygen and nitrogen gas under pressure, which was admitted through a two-way tap at the bottom of the root chamber at the rate of 1 litre per hour. This aerating gas, together with the nitrogen used for circulating the culture solution and the gases driven off from the culture solution in the boiling-tube, was collected in tube T<sub>1</sub> and allowed to pass through a spiral bubbler (S.B.), where the carbon dioxide which it contained was absorbed in 50 ml. N/4 NaOH. The pressure in the apparatus, registered by the manometer M, was maintained the same as that of the atmosphere by balancing the positive pressure of the aerating and circulating gases with a negative pressure produced by a constant head aspirator.

The apparatus was used in a greenhouse maintained at  $17 \pm 0.5^\circ \text{C}$ . The temperature of the culture solution which circulated through the apparatus was indicated by a thermometer T inserted in the circuit near the root chamber. It was maintained at  $20 \pm 0.5^\circ \text{C}$ . by controlling the temperature of the cooling water which passed through condenser E.

### *Plant material*

Barley seedlings from seeds obtained from the same sample of the variety 'Plumage Archer' were used in all the experiments. The method for germinating the seeds was similar to that used by Hoagland and Broyer (1936) except that glass dishes were used, and the zinc screens replaced by heavy mosquito-netting stretched tightly over circular dishes. The seeds were soaked in tap-water for 15 hours and then spread on the netting and allowed to germinate in tap-water in a dark incubator at  $22^\circ \text{C}$ . After 4 days, when the seedlings were about 1 in. high, they were transferred to a growth chamber and gradually exposed to the full illumination of 300 ft.-candles (200-watt Osram lamp). The temperature of the chamber was  $22 \pm 1^\circ \text{C}$ . during the light periods of 16 hours and  $18 \pm 1^\circ \text{C}$ . during the dark periods of 8 hours.

Approximately 24 hours after the seedlings were transferred to the growth chamber, fifty-eight of the most uniform were removed from the netting for assembly in the bakelite cover of the root chamber. The pericarp and endosperm were detached from each seedling in order to remove any reserve nutrient supply and to reduce the risk of fungal infection. Each seedling was then

threaded through a hole in the bakelite cover and sealed into position by dropping a molten mixture of vaseline and bees-wax round it and into the

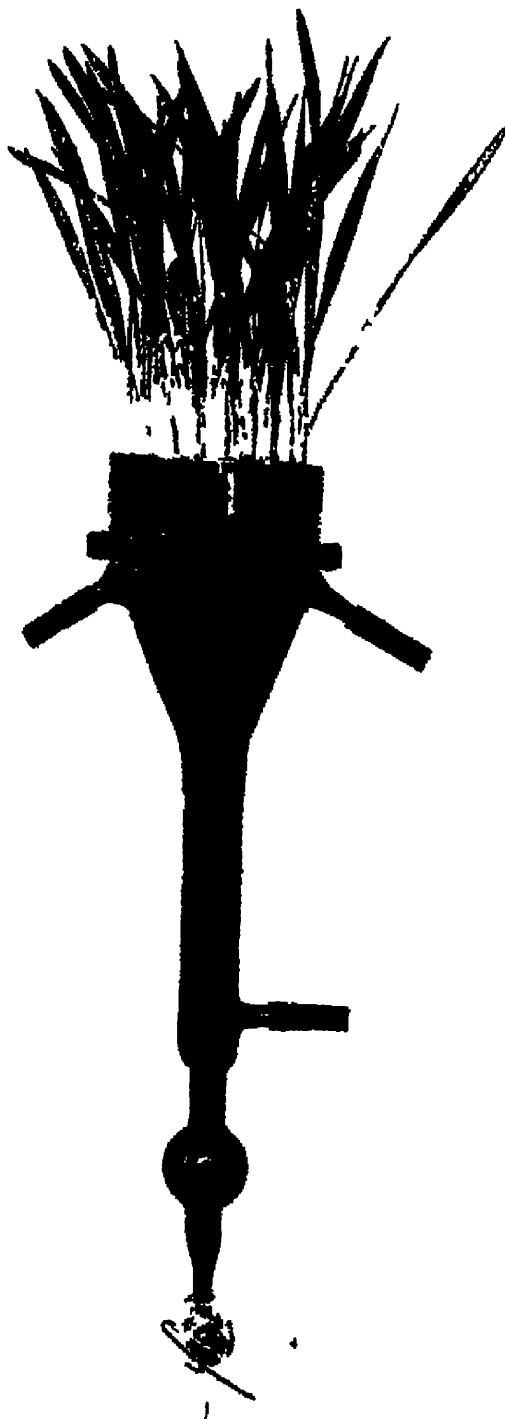


FIG. 2. Fifty-eight barley plants sealed into root chamber and ready for assembly in the apparatus. For details see text.

hole in the bakelite, from the upper and lower side. This mixture, which melted at  $35^{\circ}\text{C}$ ., formed an airtight seal and had no harmful effect on the plants.

The bakelite cover containing the fifty-eight seedlings was then supported on the rim of a 600-ml. beaker containing a dilute culture solution (5 ml. stock A + 5 ml. stock B per litre—see below) and returned to the growth chamber.

After 3 days the plants were ready for use in the apparatus. The bakelite cover was sealed to the root chamber, and a metal collar, 1 in. deep, was fitted into a groove on the upper surface of the cover. A 1.5 per cent. solution of melted agar at 35° C. was then poured into the collar and allowed to set round the stems of the plants in order to form a secondary seal between the plants and the root chamber cover (Fig. 2). This unit of fifty-eight plants was used throughout the experiment.

### Culture solution

A four-salt solution made by diluting equal quantities of the following two-stock solutions, A and B, was used for growing the standard seedlings and in all the experiments.

Stock solution A: $\text{Ca}(\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}$	0.0204 M.
$\text{KNO}_3$	0.0554 M.
Stock solution B: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.0195 M.
$\text{KH}_2\text{PO}_4$	0.0301 M.

Reagents of analytical purity were used and the stock solutions were made and diluted with 'glass-distilled' water. Hoagland's 'A-Z' solution of the following composition and a 0.5 per cent. solution of iron tartrate were added at the rate of 1 ml. per litre of culture solution.

### A-Z solution

(g. mol. per 18 litres)

$\text{Al}_2(\text{SO}_4)_3$	1.0	$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	7.0
KI	0.5	$\text{H}_3\text{BO}_3$	11.0
KBr	0.5	$\text{ZnSO}_4$	1.0
$\text{TiO}_2$	1.0	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	1.0
$\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$	0.5	$\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$	1.0
LiCl	0.5	$\text{Co}(\text{NO}_2)_2 \cdot 6\text{H}_2\text{O}$	1.0

The pH of the culture solution was approximately 6.0.

### Experimental method

In order to facilitate the comparison of different treatment effects the apparatus was duplicated. The two units (R and L) were assembled side by side, and the shoots of the two sets of plants were enclosed in a single chamber illuminated by a 200-watt 'Osram' water-cooled lamp. The light intensity in this chamber was 250 ft.-candles and the temperature  $26 \pm 1^\circ \text{C}$ .

The bakelite cover supporting the plants was sealed on to the root chamber the evening before the start of an experiment; 100 ml. of dilute culture solution (5 ml. stock A and 5 ml. stock B per litre) were added and the light above the plants turned on.

Each apparatus held 150 ml. of solution which at the start of an experiment was added through the funnel provided at the side of the root chamber. To start the liquid circulating after the gas streams had been turned on, it sufficed momentarily to close tap  $P_1$  and open tap  $P_2$  in order to force the liquid through condenser E (Fig. 1.) Once started the apparatus worked automatically.

(a) *Salt uptake.* This was estimated by analysing the culture solution

before and after contact with the roots. Five hours was taken as the standard time-interval. At the end of this period the culture solution was drained and the whole apparatus washed out with five fillings of distilled water. The culture solution, together with the wash water, was made up to 1 litre and chemical analyses carried out on aliquot portions.

Total nitrogen was estimated by using the micro-Kjeldahl apparatus of Parnas and Wagner as described by Pregl (1920). Nitrate was included by an adaptation to the micro-scale of the reduced iron method of Pucher, Leavenworth, and Vickery (1930). Nitrate nitrogen was estimated by the phenol-disulphonic acid method; a Hellige colorimeter was used and the procedure followed was essentially that described by Ashton (1935). Phosphorus was determined by the colorimetric method described by Holman and Pollard (1937). The method is a modification of the molybdenum-blue colorimetric method for phosphate using stannous chloride for reduction. A Hellige colorimeter was used and fresh standards made for each comparison. Potassium was determined by a micro-colorimetric method described by Sideris (1937).

(b) *Root respiration.* This was estimated by measuring the quantity of carbon dioxide liberated by the roots over four successive hour intervals. The duration of each experiment was usually 5 hours, but respiration measurements were not started until the end of the first hour in order to ensure that any carbon dioxide originally present in the apparatus or the culture solution had been driven off. The carbon dioxide liberated from the culture solution in the boiling-tube together with any carried over by the circulating and aerating gases was absorbed in N/4 sodium hydroxide and estimated by an electrical conductivity method described by Newton (1935) and Heath (1939). The conductivity cell was calibrated by absorbing known quantities of carbon dioxide (liberated by the decomposition of a known amount of pure sodium carbonate) in 50 ml. of N/4 NaOH of known electrical conductivity. The change in the conductivity of the NaOH was measured and a concentration-conductivity curve plotted. The results are presented in Table I. Each result is the mean of two determinations, the difference between which was never greater than 1 per cent. of the mean. These values can be represented adequately by a straight line passing through the origin.

TABLE I

*The Relationship between Mg. Carbon Dioxide absorbed and the Change in Conductivity of 50 Ml. of N/4 NaOH*

Carbon dioxide (mg.).	Resistance (ohms).	Conductivity ( $\times 10^4$ ).	Change in conductivity ( $\times 10^4$ ).
0	302.67	3.3039	—
13.502	316.69	3.1576	0.1463
27.004	334.70	2.9877	0.3162
54.007	369.10	2.7092	0.5947
81.011	416.58	2.4005	0.9034
108.01	473.00	2.1142	1.1897

(c) *Root respiration.* The concentration of oxygen in the culture solution, which was controlled by the percentage of oxygen in the aerating stream, was determined by the Winkler method, using 10-ml. syringe pipettes. The design of the pipettes and their application to this method have been described by Krogh (1935, 1935a). An accuracy of  $\pm 0.01$  ml. of oxygen per litre of solution was obtained.

EXPERIMENTAL DATA

*The effect of varying the oxygen tension and nutrient concentration of the culture solution on root respiration and nitrate uptake.*

The first experiments carried out were of an exploratory nature and were intended to assess the accuracy with which results could be obtained. In the course of this preliminary work various modifications were made to the apparatus, which in its final form has already been described.

These experiments showed that the apparatus worked well and that the analytical methods were adequate. A summary of this preliminary work on the effects of varying the oxygen tension and nutrient concentration of the culture solution is given in Table II. The results have been compiled from isolated experiments, so that individual values are based upon varying numbers of observations. Nevertheless very definite trends are apparent. These are shown graphically in Fig. 3, in which respiration and nitrate uptake, at various oxygen tensions, expressed as a percentage of the highest value, have been plotted against nutrient concentration.

TABLE II  
*Respiration and Nitrate Uptake at Different Oxygen Tensions and Nutrient Concentrations of the Culture Solution*

Nutrient concentration.		Respiration			Nitrate uptake		
Relative.	p.p.m. N.	mg. CO <sub>2</sub> /hr. per g. dry wt. roots.			mg./N/hr. per g. dry wt. roots.		
0	0	—	—	9.99	0	0	0
2	32.4	9.37	10.84	14.42	0.619	0.667	0.897
4	64.8	9.45	12.06	14.51	0.731	1.27	1.40
6	97.2	9.58	—	—	1.05	1.59	—
8	130	9.76	13.64	—	1.12	2.06	—
Oxygen tension		0%	20%	40-100%	0%	20%	40-100%

At all oxygen levels, including anaerobic conditions, the uptake of nitrate increases with the concentration, but the slopes of the curves vary according to the oxygen supply. This constitutes an interaction between these factors. It will be noted that a horizontal line through the final point on the anaerobic curve cuts the other curves at points corresponding with lower concentrations of nutrient. It appears, therefore, that for the same rate of nitrate uptake varied combinations of the factors studied give similar results. If such a

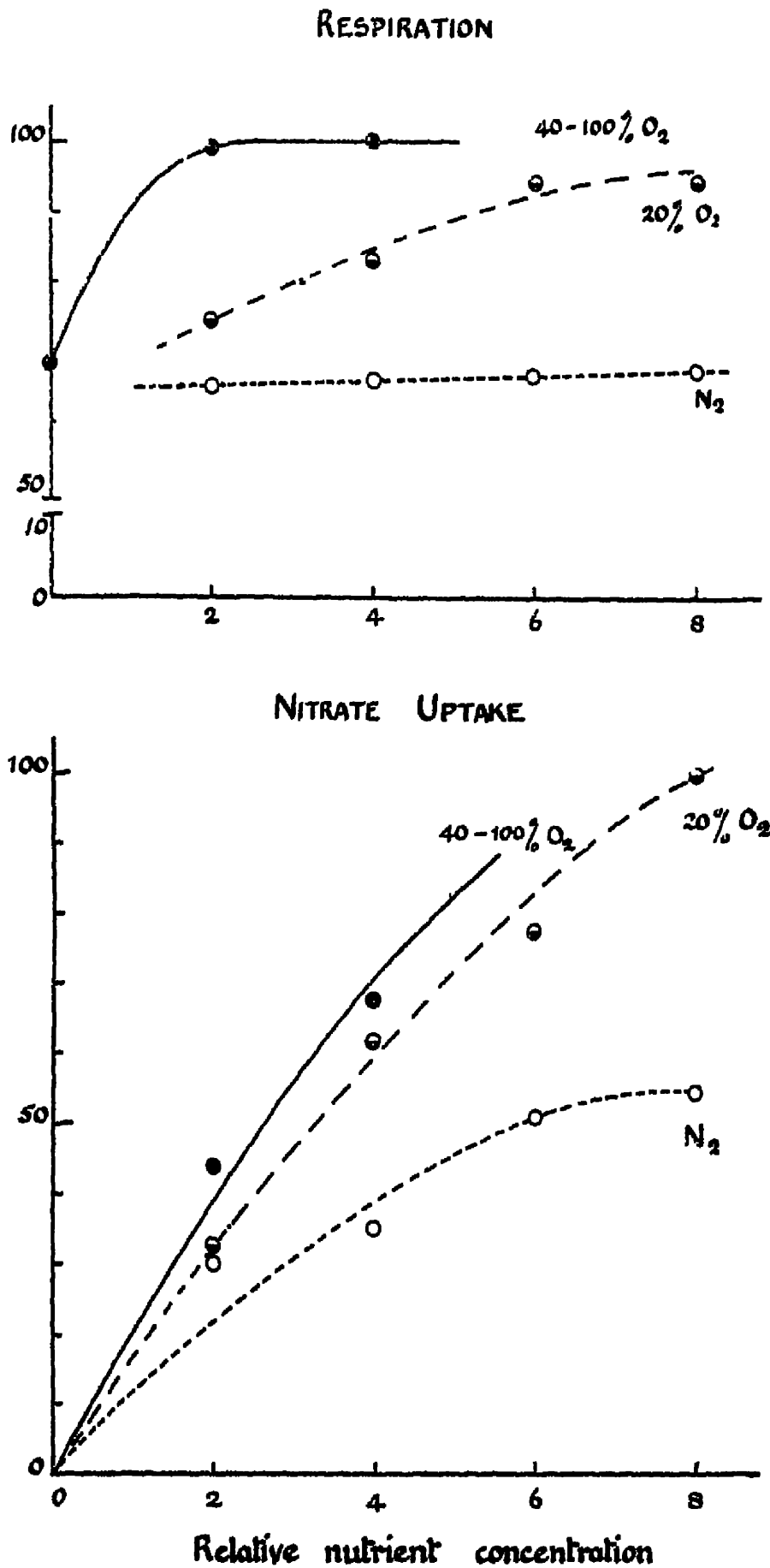


FIG. 3. Preliminary results of respiration and nitrate uptake by fifty-eight plants, at different oxygen tensions and nutrient concentrations, expressed as a percentage of the highest value.

relation held for long periods of growth as well as for short-term experiments, it would indicate that nitrate accumulation in a given time under anaerobic conditions could be brought to the same level as under aerobic conditions by merely increasing the concentration of the nutrient solution. This result seemed sufficiently important to test directly, for which purpose the following growth experiments were performed.

*Growth of barley in anaerobic and aerobic nutrient cultures*

It was assumed that nitrogen being the main nutrient factor in controlling

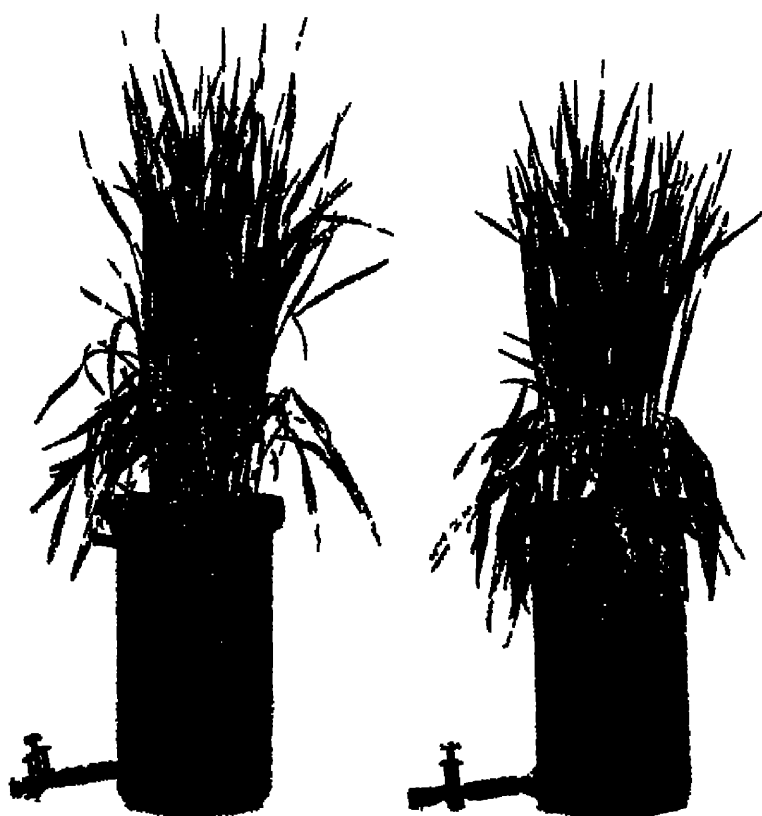


FIG. 4 Photograph of two sets of barley plants grown for 12 days in culture solutions of different oxygen tension and nutrient concentration. The plants on the left were grown in a culture solution of relative nutrient concentration 8 through which nitrogen gas was bubbled continuously. The plants on the right were grown in a similar culture solution of relative concentration 2 through which oxygen gas was bubbled continuously.

growth, the uptake of equal quantities of nitrate by the plants would be associated with equal growth rates. Selecting combinations of factors to give equal rates of nitrate uptake and growing plants under these conditions should result in equal growth in equal time. From the values in Fig. 3 two combinations were selected, namely, pure nitrogen and relative nutrient concentration 8, and pure oxygen and relative concentration 2.

Six-hundred-millilitre Pyrex beakers were used as culture vessels. They were painted black on the outside and fitted with inlet and outlet tubes for aerating gases and nutrient solutions. The aerating gas was admitted into the solution from a tapered glass tube opening at the bottom of the beaker

and escaped through a narrow-bore glass tube at the top of the beaker. The rim of the beaker was ground flat and fitted on to a bakelite disk which held the plants. The plants were sealed into the bakelite disk in the manner previously described and the bakelite disk was sealed to the beaker with plasticine.

In the first experiment two culture vessels were used and twenty-seven standard barley plants, grown as described in the first part of this paper, were sealed into each.

Vessel A was filled with a culture solution of relative nutrient concentration 8 containing 130 p.p.m. nitrogen (20 ml. stock A+20 ml. stock B per litre). Nitrogen gas, from which the last traces of oxygen had been removed by passage through a bead tower containing pyrogallol, was bubbled through the solution continuously.

Vessel B contained a culture solution of relative nutrient concentration 2 containing 32 p.p.m. nitrogen (5 ml. stock A+5 ml. stock B per litre) through which oxygen gas was bubbled at the same rate as the nitrogen was passed through vessel A.

The plants were kept in a greenhouse and the solutions in both vessels renewed every 2 days. Great care was taken not to allow any oxygen to enter vessel A when the solutions were changed. The diluted stock solution was stored under anaerobic conditions and the culture vessel was refilled by first forcing the old solution out with nitrogen gas.

The experiment was started on July 9 and the plants were removed for weighing on July 16 (7 days). Two hours after the apparatus and plants were assembled there was a very marked exudation from the hydathodes of the plants in vessel B which was receiving oxygen, but none from the plants in vessel A.

At the end of the experiment the plants in both vessels were healthy, but those which had received oxygen (vessel B) appeared more vigorous. Their roots were longer and more branched than those in vessel A. The weights of the shoots and roots of both sets of plants are shown in Table III.

The second experiment was carried out in exactly the same manner. Fifty-six plants were used in each vessel and the experiment was continued for 12 days (July 16-28). In this experiment the first leaf of the plants that were receiving nitrogen started to die from the tip sooner than the first leaves of the plants receiving oxygen. The weights of the shoots and roots of both sets of plants are given in Table III, and Fig. 4 portrays the plants at the end of the experiment.

The results show that it is possible to grow barley plants for a considerable time in a culture solution devoid of oxygen, and that growth (as measured by weight) in such a solution was very little less than in one aerated with oxygen, when the nutrient concentration of the former was four times that of the latter.

The most noticeable difference in the plants grown under the two conditions was in root growth. The plants growing under anaerobic conditions

TABLE III

*Weight of Barley Plants grown in Anaerobic and Aerobic Culture Solutions*

Experi- ment no.	Treatment.			Duration (days).	Plants (no).	Shoot wt. g.		Root wt. g.	
	Aerating gas.	Rela- tive.	Nutrient concentration. Milli- equiv. NO <sub>3</sub> .			Fresh.	Dry.	Fresh	Dry.
1A	N <sub>2</sub>	8	9.26	7	27	4.419	0.420	1.762	0.116
1B	O <sub>2</sub>	2	2.32	7	27	5.469	0.485	2.303	0.151
2A	N <sub>2</sub>	8	9.26	12	56	15.93	1.69	5.96	0.429
2B	O <sub>2</sub>	2	2.32	12	56	15.63	1.43	6.67	0.589

appeared to develop their leaves faster, but these were not so large and began to die quicker than the corresponding leaves on the plants grown in the aerated culture solution.

This experiment thus confirmed the previous estimate of NO<sub>3</sub> uptake and the interaction of nutrient concentration and oxygen supply.

In view of the large amount of work which has been carried out with excised root systems it seemed desirable to investigate the behaviour of the standard plants used before and after removal of the aerial organs. This part of the work remains quite incomplete. Sugar analyses of the plant tissues and analyses of the expressed sap would be necessary to determine the part played by carbohydrate in maintaining the respiration and the process of accumulation in the plant tissues in the presence and absence of leaves. This work was interrupted before such analyses could be undertaken so that the only data available are carbon dioxide evolved and nitrogen uptake. None the less the data are not without interest.

*The effect of the removal of the aerial organs of barley plants on root respiration and the uptake of nitrate*

Two sets of fifty-eight standard barley plants were assembled in two units of the apparatus, L and R, as previously described. Oxygen was used as the aerating gas and gave a concentration in the solution of approximately 8 ml. per litre (N.T.P.). Both sets of plants were given exactly the same treatments, except that at the end of the first 5 hours the shoots of the plants in root chamber R were cut off at the surface of the bakelite disk and the cut ends of the plants covered with melted agar. The experiment was continued for 31 hours and five consecutive measurements of respiration and nitrate uptake recorded. At the end of the first experimental period the nutrient concentration of the culture solution was increased from 28 to 107 parts per million of nitrogen.

The results are presented in Table IV. In Fig. 5 the respiration and nitrate uptake rates are plotted at the times representing the midpoints of the experimental periods.

The respiration rate of the roots of the whole plants was not affected appreciably by the increase in the concentration of the culture solution and increased at a steady rate throughout the experiment. The uptake of nitrate

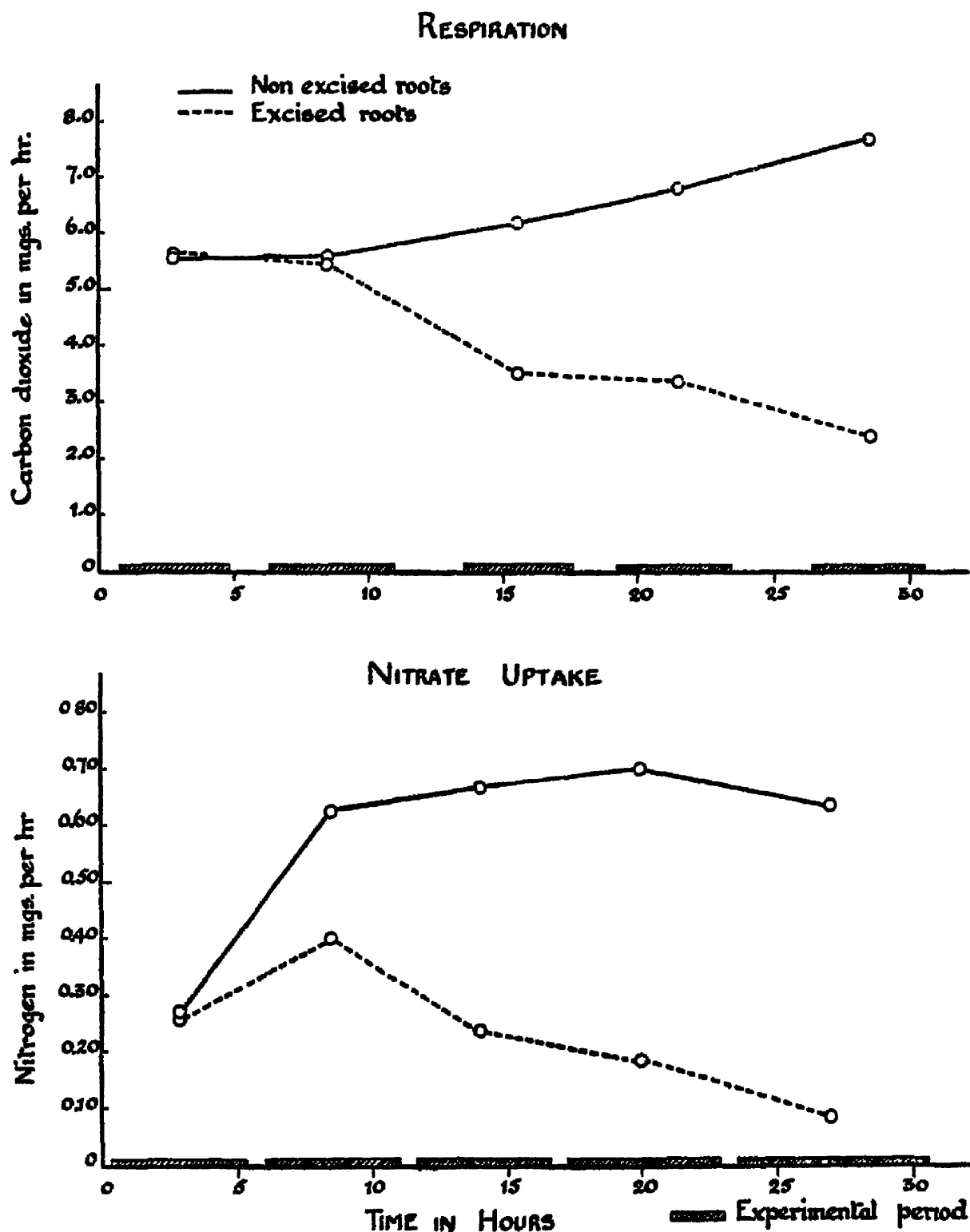


FIG. 5. Changes in respiration rate and nitrate uptake with time for whole plants and excised roots.

increased in response to the increase of nitrate in the culture solution and during the remainder of the experiment remained fairly constant.

The removal of the shoots caused an immediate fall in nitrate uptake but did not affect respiration until the third experimental period,  $5\frac{1}{2}$  hours after

TABLE IV

*Trend of Respiration and Nitrate Uptake Rates with Time for  
58 Barley Plants, with and without Shoots*

Nutrient concentration. p.p.m. nitrogen.	Experimental period (hrs.)	Respiration (CO <sub>2</sub> mg per hour).		Nitrogen uptake (N mg. per hour).	
		Root Chamber		Root Chamber	
		L.	R.	L.	R.
28	5.0	5.54	5.62 shoots removed	0.264	0.256 shoots removed
107	5.0	5.60	5.46	0.625	0.400
107	5.0	6.20	3.51	0.666	0.238
107	5.5	6.81	3.04	0.700	0.184
107	7.0	7.69	2.41	0.637	0.084

excision, after which time decreases in respiration and in nitrate uptake occurred.

An examination of the roots at the end of the experiment showed that the roots of the whole plants had grown more during the experiment than the excised roots. They were longer and more branched. The respective weights were:

	Fresh wt. g.	Dry wt. g.
Whole plant roots . . .	5.65	0.383
Excised roots . . .	4.89	0.257

The difference of 0.128 g. in dry weight is significant (S.E. = 0.0203, Table V).

The results show that both root respiration and nitrate uptake were more constant for whole plants than for those in which shoots had been removed.

The immediate decline in nitrate uptake followed by a later fall in respiration rate suggests that respiration is dependent on the uptake of nitrate and not vice versa.

#### *Factorial experiment with varying levels of oxygen tension and nutrient concentration*

(i) *Design of experiment.* Four oxygen levels and four nutrient concentrations were employed.

By aerating with pure nitrogen and mixtures with 5, 10, and 20 per cent. oxygen, concentrations of 0, 1.2, 2.4, and 4.8 ml. oxygen per litre (N.T.P.) respectively were maintained in the culture solution. These oxygen levels are referred to as A, B, C, and D.

The four nutrient concentrations, designated 1, 2, 4, and 8, were prepared by mixing equal volumes of stock solutions A and B of 2.5, 5, 10, and 20 ml. and diluting to 1 litre. Details of these stock solutions have already been given.

Combinations of the four oxygen levels with the four nutrient concentrations gave in all sixteen combined treatments.

Nutrient concentration.		Oxygen levels (ml. O <sub>2</sub> /litre).			
(milli. atmos.).	Rel.	A (0).	B (1.2).	C (2.4).	D (4.8).
24.52	8	A8	B8	C8	D8
12.26	4	A4	B4	C4	D4
6.31	2	A2	B2	C2	D2
3.16	1	A1	B1	C1	D1

For the experiment only two units of the apparatus (R and L) were available. Previous experience had shown that it was possible to use each single batch of plants for two consecutive treatments, and thus with the equipment available four of the treatments could be carried out on a single day. These considerations involved two difficulties: (1) the variation of the plants from day to day, as the two batches used each day alone could be drawn from a single sample; and (2) the effect of the previous on the following treatment, which often involved radical changes in concentration and oxygen tension which were likely to have minor effects. Replication of treatments was essential for the estimation of the errors of the experiment. This was achieved by a fourfold repetition of the sixteen combined treatments, thus constituting four 'blocks' of treatments. Each block, consisting of four by four combinations of treatments of oxygen tension and nutrient concentration, was arranged as a 'Graeco-Latin' square in which each level of each factor occurred once only in each row and column. The arrangement of the treatments in the blocks is shown in Fig. 6. The four treatments represented in any column of a block were carried out on a single day from a single batch of plants, two each in each unit R and L.

The entries occurring in corresponding columns of each block comprised the same four treatments, but were carried out in each case in a different order. The corresponding sets of columns thus constitute Latin squares of four treatments. The design has thus ensured complete balance so far as 'order of treatment' is concerned. It is not possible, however, to estimate from the sets of columns the errors due to the 'order of treatment' as in each block the four treatments constituting a column were carried out with a single batch of plants which varied from day to day. The symmetry of the design so far as sets of rows is concerned is greater. Each of the corresponding sets of rows in the four blocks constitute a 'Graeco-Latin' square of treatments. Any one set of rows is thus carried out on sixteen independent groups of plants. The four sets of 'Graeco-Latin' squares are, however, not completely independent, as on each day four treatments, one in each 'Graeco-Latin' square of rows, was carried out on the same batch of plants. From these four sets of 'Graeco-Latin' squares, however, the variance due to the 'order of treatment' can be estimated.

The design of the experiment has thus ensured a very great measure of balance of the treatments.

(ii) *Experimental procedure.* The design of the experiment having been completed, the procedure adopted to carry it into effect was as follows. One of the blocks was drawn at random. There were then four sets of four

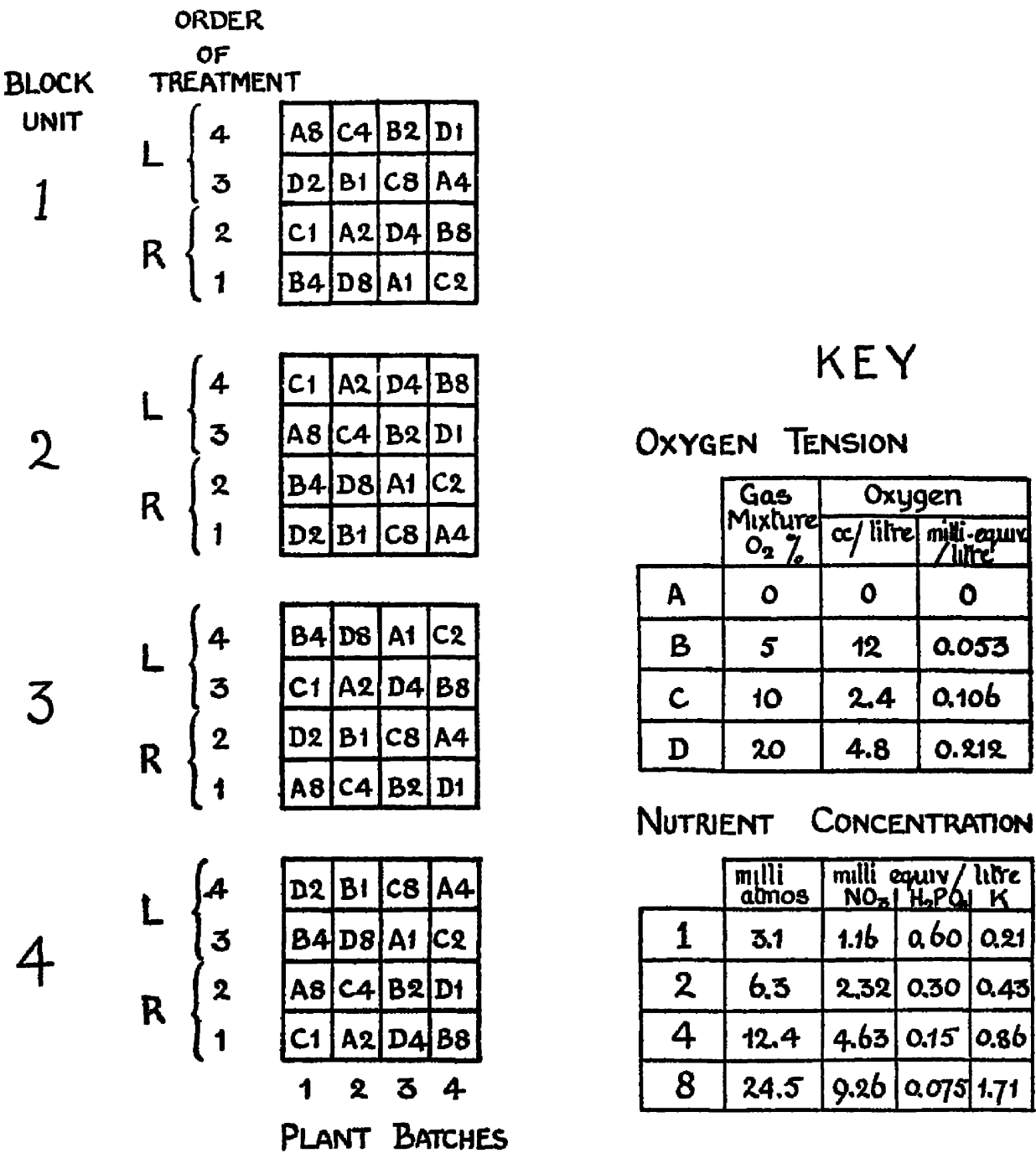


FIG. 6. Diagram showing arrangement of the 16 treatments into 4 blocks, and the order of carrying out the treatments in each unit (L and R) of the apparatus on the 16 different batches of plants.

treatments to be carried out on consecutive days. The order of days was again determined at random and represents the four columns in the block. The order of carrying out the four treatments was predetermined in the design and is shown in the columns in the diagram (Fig. 6). The convention was adopted of carrying out 'order of treatment' 1 and 2 in the right-hand (R) apparatus and 3 and 4 in the left (L).

(iii) *Analysis of variance.* The design of the experiment imposes limitations

on the analysis of variance. Owing to the need to carry out the four treatments on the same batch of plants and two consecutive treatments on the same plants it is not possible to separate and estimate the errors of all the possible interactions. The chief interest of the experiment centred in the main effects of nutrient concentration and oxygen tension and their interaction. From the four blocks the plant variance could also be estimated, and from the four 'Graeco-Latin' squares corresponding to the sets of rows (one drawn from each block) the variance due to 'order of treatment' could be calculated. As the 'order' variance was statistically quite insignificant, the variance from this cause was added to 'error' variance in all the analyses of variance carried out. The block variance also failed to reach the significant level, but as it did not fall far short for the dry-weight data it was thought best to separate the variance due to this cause from 'error' in all the analyses of variance.

(iv) *Data and statistical analyses*

(a) *Dry weight.* At the end of each day's experiments the plants were removed from the apparatus and separated into roots and shoots. The two portions were dried at 100° C. for 24 hours and then weighed. The results for each group of fifty-eight plants are given in Table V. They are presented

TABLE V  
*Dry Weight (g.) of Plants separated into Roots and Shoots*

			Plant batches.				
	Block.	Apparatus unit.	1.	2.	3.	4.	Mean.
Roots	1	R	0.218	0.273	0.251	0.255	0.249
		L	0.223	0.268	0.262	0.241	
	2	R	0.255	0.235	0.266	0.261	0.259
		L	0.256	0.231	0.278	0.271	
	3	R	0.277	0.296	0.247	0.241	0.266
		L	0.288	0.276	0.254	0.245	
	4	R	0.305	0.242	0.280	0.269	0.258
		L	0.292	0.242	0.276	0.262	
Standard error 0.0203 (7.8 %)							
Shoots	1	R	1.114	0.990	1.206	1.200	1.109
		L	1.076	1.050	1.126	1.112	
	2	R	0.806	0.840	0.824	0.970	0.857
		L	0.804	0.792	0.882	0.941	
	3	R	1.100	1.056	1.216	0.995	1.104
		L	1.177	1.135	1.198	0.957	
	4	R	1.100	1.195	1.354	1.089	1.167
		L	1.134	1.164	1.231	1.072	
Standard error 0.152 (14.3 %)							



TABLE VII

Salt-uptake Data: Mg. Nutrient per Hour by 58 Plants. Nitrogen as N, Phosphorus as P<sub>2</sub>O<sub>5</sub>, Potassium as K.

Relative nutrient conc.	Block no.	0%				5%				10%				20%									
		Order expt.	N.	P.	K.	Order expt.	N.	P.	K.	Order expt.	N.	P.	K.	Order expt.	N.	P.	K.						
8	4	3	0.088	0.224	0.382	1	0.456	0.176	1.108	4	0.724	0.184	1.182	2	0.232	0.168	1.208						
	2	2	0.544	0.250	0.652	4	0.328	0.144	1.228	1	0.623	0.260	1.206	3	0.684	0.376	1.182						
	3	1	0.152	0.256	0.474	2	0.704	0.180	1.034	3	0.736	0.152	1.260	4	0.684	0.208	1.272						
	1	4	0.400	0.336	0.488	3	0.608	0.124	1.122	2	0.408	0.064	1.176	1	0.872	0.184	1.214						
4	4	4	0.228	0.158	0.304	2	0.236	0.134	0.572	3	0.352	0.144	0.568	1	0.290	0.160	0.626						
	2	1	0.216	0.160	0.186	3	0.490	0.115	0.578	2	0.320	0.152	0.620	4	0.500	0.128	0.622						
	3	3	0.236	0.164	0.352	4	0.264	0.160	0.620	1	0.248	0.208	0.636	2	0.300	0.216	0.644						
	1	2	0.400	0.264	0.154	1	0.400	0.104	0.614	4	0.236	0.168	0.608	3	0.344	0.132	0.660						
2	4	1	0.070	0.092	0.022	3	0.186	0.092	0.328	2	0.174	0.076	0.274	4	0.174	0.126	0.280						
	2	4	0.096	0.124	0.0	2	0.264	0.084	0.324	3	0.136	0.080	0.274	1	0.229	0.108	0.320						
	3	2	0.110	0.204	0.0	1	0.204	0.108	0.320	4	0.224	0.088	0.292	3	0.139	0.052	0.288						
	1	3	0.266	0.092	0.324	4	0.268	0.064	0.312	1	0.238	0.080	0.284	2	0.232	0.068	0.294						
1	4	2	0.066	0.055	0.030	4	0.124	0.057	0.108	1	0.056	0.053	0.110	3	0.134	0.057	0.122						
	2	3	0.148	0.060	0.086	1	0.070	0.045	0.160	4	0.347	0.053	0.142	2	0.156	0.059	0.094						
	3	4	0.150	0.050	0.0	3	0.103	0.053	0.158	2	0.192	0.046	0.100	1	0.120	0.052	0.118						
	1	1	0.150	0.053	0.032	2	0.148	0.059	0.116	3	0.198	0.061	0.088	4	0.154	0.046	0.106						
Oxygen tension																							
A 0%						B 5%						C 10%						D 20%					

TABLE VIII  
*Respiration Data: Analysis of Variance*

	Degrees of freedom.	Mean square.
Blocks . . . . .	3	0.216
Oxygen tension . . . . .	3	2.573*
Nutrient concentration . . . . .	3	0.439†
Oxygen × nutrient . . . . .	9	0.061
Error . . . . .	45	0.134

Standard error (of total of 4) = 0.231 (18.3 %).

\* = Significant 1 % point.      † = Significant 5 % point.

For each level of oxygen tension, there corresponds a value for carbon dioxide production which is almost independent of the nutrient concentration. The mean carbon dioxide production for varying levels of nutrient is almost constant and the only significant departure is that at the lowest nutrient concentration. It is not surprising, therefore, that no significant interaction has been established and that the increase in respiration with increasing oxygen supply is the same at all levels.

The differences referred to are in no way related to variations in the weight of the plants.

(c) *Salt uptake.* The complete results for all three nutrients studied are given in Table VII. The average values together with the three-dimensional diagrams drawn from them are shown in Figs. 7, 8, and 9. An analysis of variance of the results for each nutrient is given in Table IX.

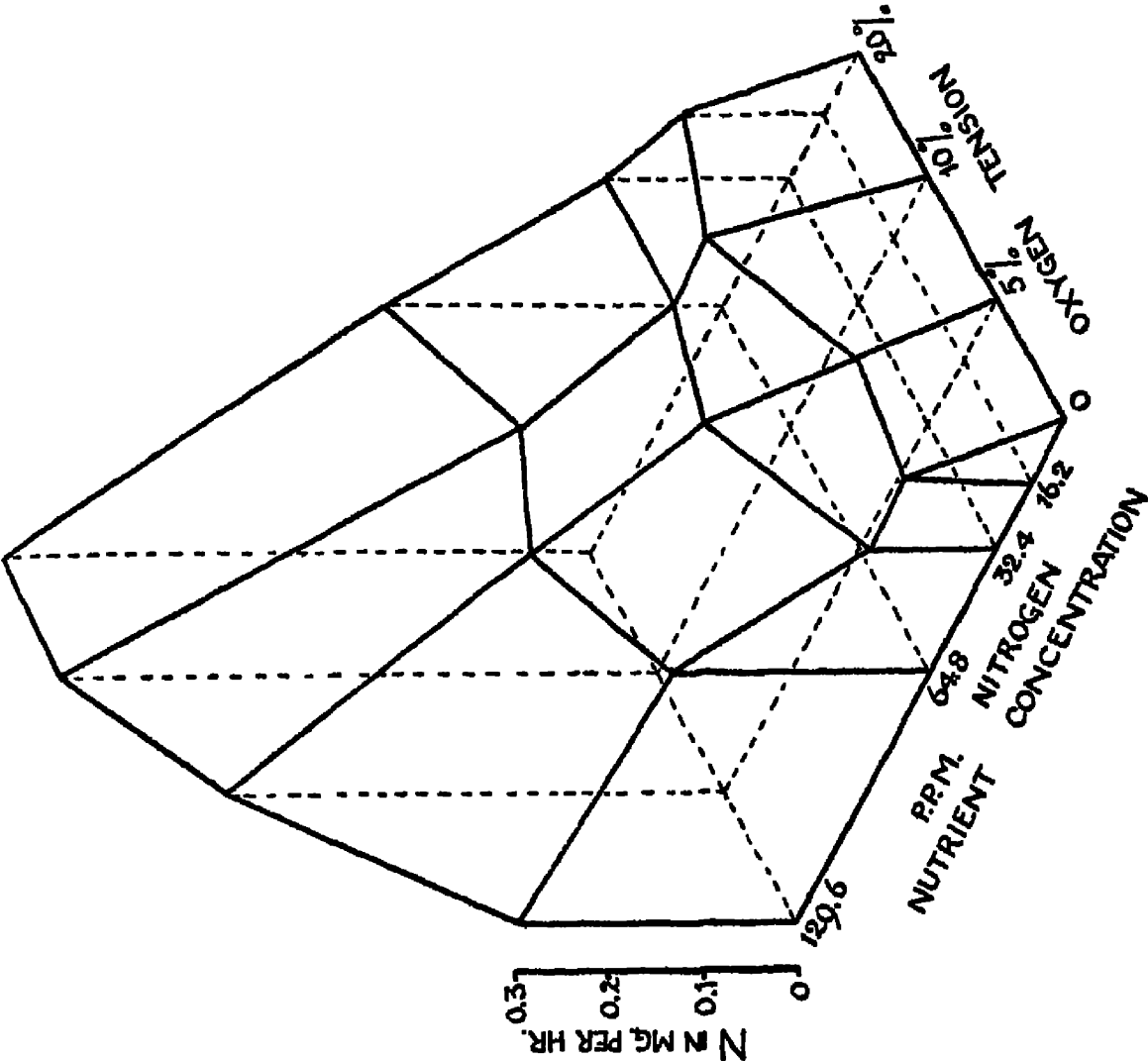
TABLE IX  
*Salt-uptake Data: Analyses of Variance*

	Degrees of freedom.	Mean squares.		
		Nitrate.	Phosphate.	Potassium.
Blocks . . . . .	3	0.03801	0.001553	0.00026
Oxygen tension . . . . .	3	0.05139*	0.008415†	0.08915†
Nutrient concentration . . . . .	3	0.44266†	0.072396†	0.43609†
Oxygen × nutrient . . . . .	9	0.02054	0.002430	0.01411†
Error . . . . .	45	0.01315	0.001550	0.000862
σ (Total of 4) . . . . .		0.229 (19.7 %)	0.0787 (15.3 %)	0.0587 (3.1 %)

\* = Significant 5 % point.      † = Significant 1 % point.

Graphical representations of the effects of the factors studied are shown as perspective models of the interaction surfaces in Figs. 7, 8, and 9. The contours of these surfaces are drawn through the mean experimental values and show the relation of uptake to increasing oxygen tension and nutrient concentration respectively. The surfaces for nitrate and potassium resemble each other more closely than that for phosphate.

The highly significant main effect of oxygen tension shows that in every case the contours parallel to the oxygen axis depart significantly from horizontal

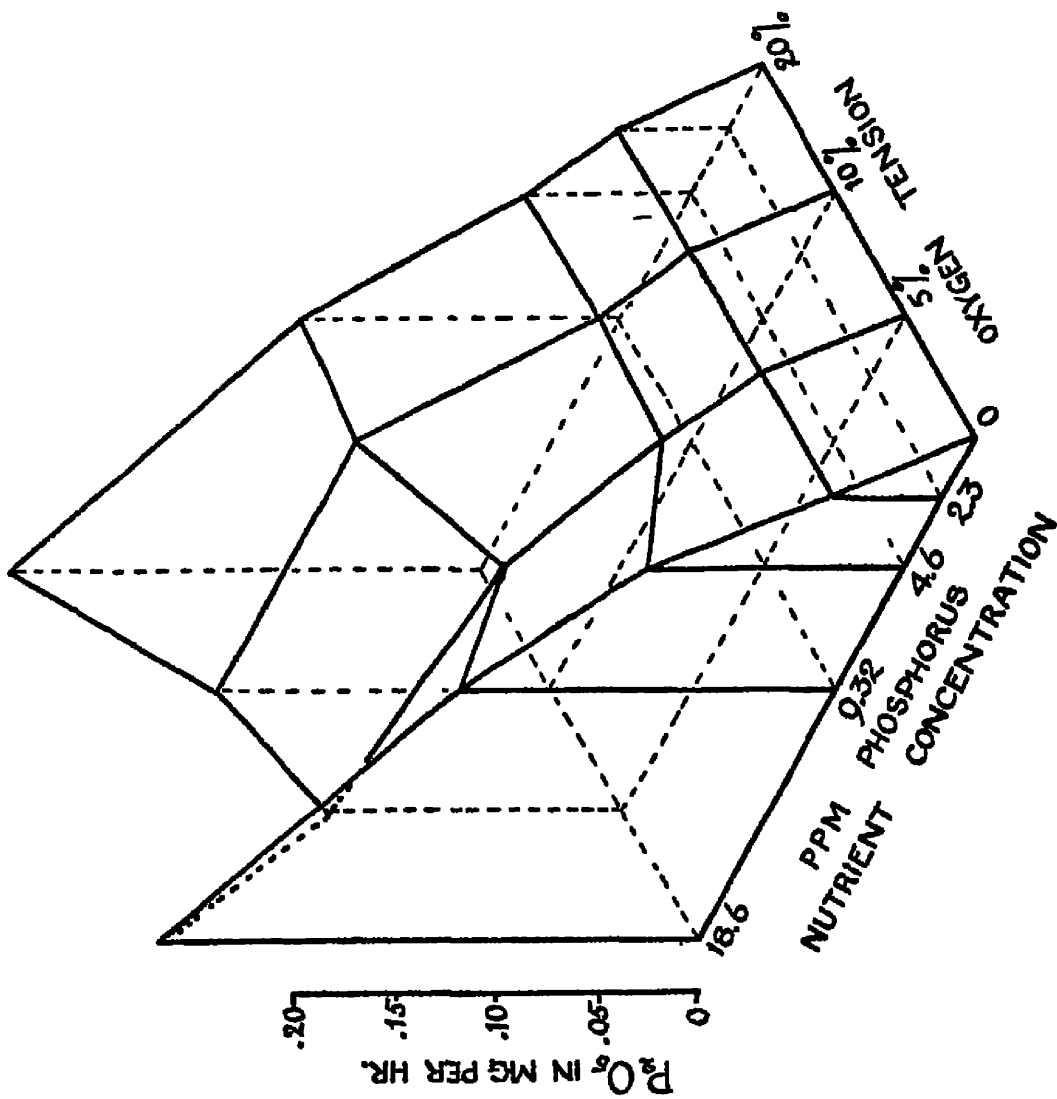


1. NITRATE  
Mg. N per hr. per 58 Plants

RELATIVE NUTRIENT CONC.	OXYGEN TENSION				MEAN
	A 0	B 5%	C 10%	D 20%	
8	0.296	0.524	0.625	0.618	0.516
4	0.270	0.348	0.289	0.359	0.316
2	0.130	0.231	0.194	0.191	0.186
1	0.129	0.111	0.198	0.141	0.145
MEAN	0.206	0.304	0.326	0.327	

$\sigma$  (TOTAL OF 4) = 0.229

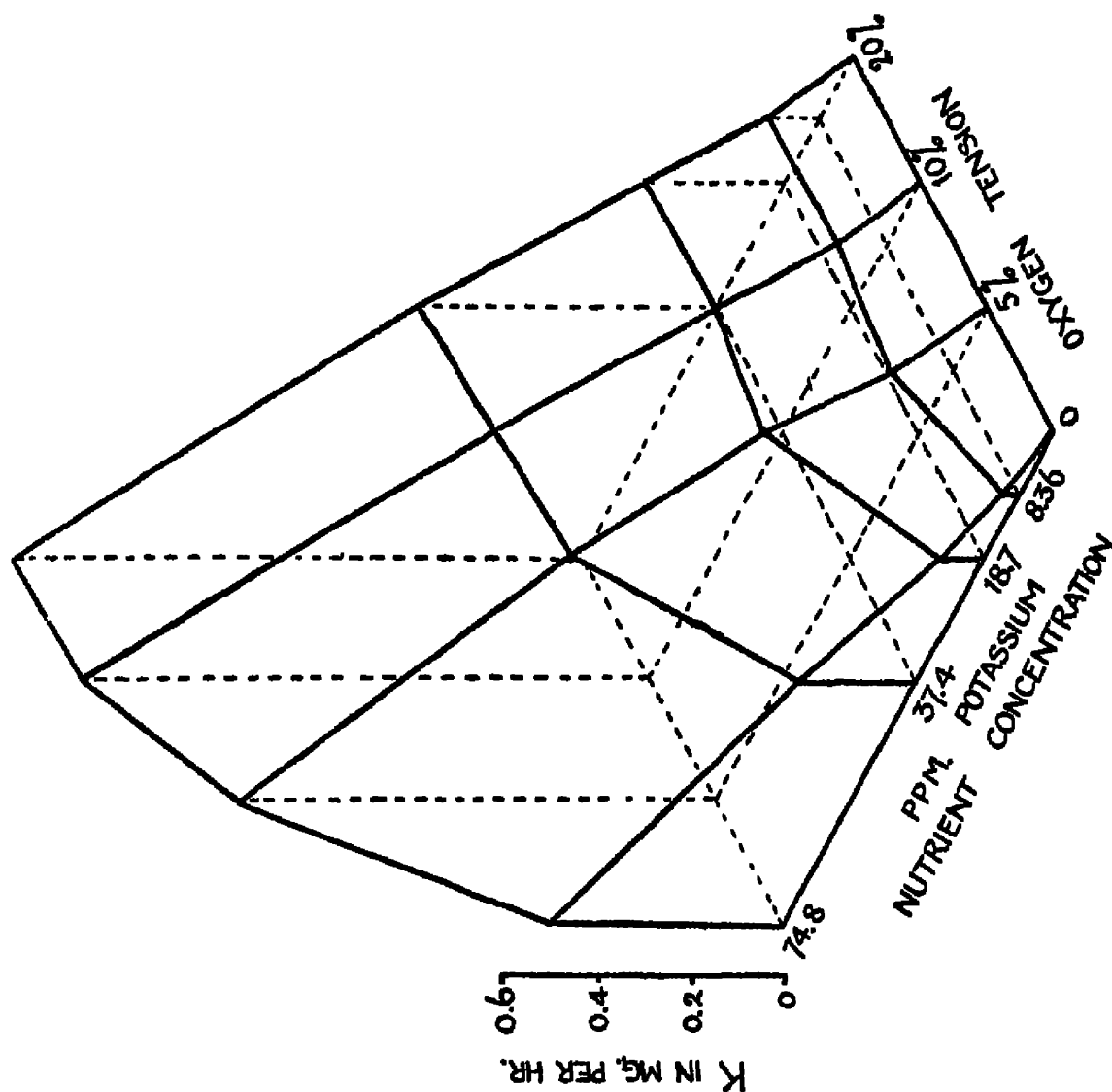
Fig. 7. Nitrogen absorbed at various oxygen tensions and nutrient concentrations (58 plants).



RELATIVE NUTRIENT CONC	OXYGEN TENSION				MEAN
	A 0	B 5%	C 10%	D 20%	
8	0.267	0.156	0.165	0.234	0.206
4	0.187	0.128	0.168	0.159	0.160
2	0.128	0.087	0.081	0.089	0.096
1	0.055	0.054	0.053	0.054	0.054
MEAN	0.159	0.106	0.117	0.114	

$\sigma(\text{TOTAL OF 4}) = 0.079$

FIG. 8. Phosphorus ( $P_2O_5$ ) absorbed at various oxygen tensions and nutrient concentrations (58 plants).



RELATIVE NUTRIENT CONC.	OXYGEN TENSION				MEAN
	A 0	B 5%	C 10%	D 20%	
8	0.499	1.123	1.206	1.219	1.011
4	0.249	0.596	0.608	0.638	0.523
2	0.087	0.321	0.281	0.298	0.247
1	0.037	0.136	0.110	0.110	0.098
MEAN	0.218	0.544	0.551	0.567	

Fig. 9. Potassium absorbed at various oxygen tensions and nutrient concentrations (58 plants).

lines and establish the increasing uptake as oxygen supply is increased from 0 to 20 per cent. A marked difference is, however, seen between the various nutrients. With nitrate and potassium the uptake rises throughout the range of oxygen tension with the greatest slope of the surfaces between 0 and 5 per cent. oxygen. At higher oxygen tensions the values tend towards a constant so that the contour lines resemble hyperbolae. In the case of phosphate, however, the surface shows a marked trough at 5 per cent. oxygen tension and the uptake rate at 20 per cent. is always less than in pure nitrogen. This striking effect is highly significant.

The main effect of nutrient concentration is in all instances highly significant and in no case has the uptake reached a maximum over the range of concentration used. The contour lines parallel to the nutrient axis are linear for potassium, indicating a proportionality between uptake and external concentration. In the case of nitrate and phosphate there is throughout the range evidence of a departure from proportionality which is greatest for nitrate under anaerobic conditions and tends to disappear at 20 per cent. oxygen. In the case of phosphate the contours are also curvilinear, the effect being greatest at 5 per cent. oxygen.

So far as the interaction of oxygen tension and nutrient concentration is concerned, only in the case of potassium does this attain statistical significance when all the data are used. The type of interaction displayed has already been indicated, namely, in the case of potassium and nitrate it consists in the increasing slopes of the contours parallel to both nutrient and oxygen axes as the levels are raised, and in the case of phosphate to the variable slope of the contours parallel to the nutrient axis and the deepening of the trough parallel to the oxygen axis.

The effects of oxygen tension are obviously greatest between 0 and 5 per cent., and analyses of variance carried out for these values only showed that the main effects and interaction were now highly significant for nitrate as well as potassium though the interaction for phosphate still failed to reach significance.

(d) *Relation between respiration and salt uptake.* The relation between carbon dioxide and salt uptake, for each of the ions  $\text{NO}_3^-$ ,  $\text{H}_2\text{PO}_4^-$ , and  $\text{K}^+$ , is shown in Fig. 10. In these graphs the curves for the four nutrient levels are given in each case as milli-equivalents per hour for fifty-eight plants, plotted against milligrams carbon dioxide per hour. These curves resemble somewhat the contours of the solids in Figs. 7–9, parallel to the oxygen axis. At the lowest nutrient level for all ions the uptake is almost independent of the carbon dioxide produced, but with higher nutrient levels in the case of nitrate and potassium the uptake rises with carbon dioxide production tending towards a maximum value, so that the curves again resemble hyperbolae. With phosphate there is much less evidence of a relation between the factors, and with rising phosphate concentration a minimum uptake rate occurs in the middle of the carbon dioxide range. This set of curves shows that at all respiration rates the uptake increases with the concentration, and

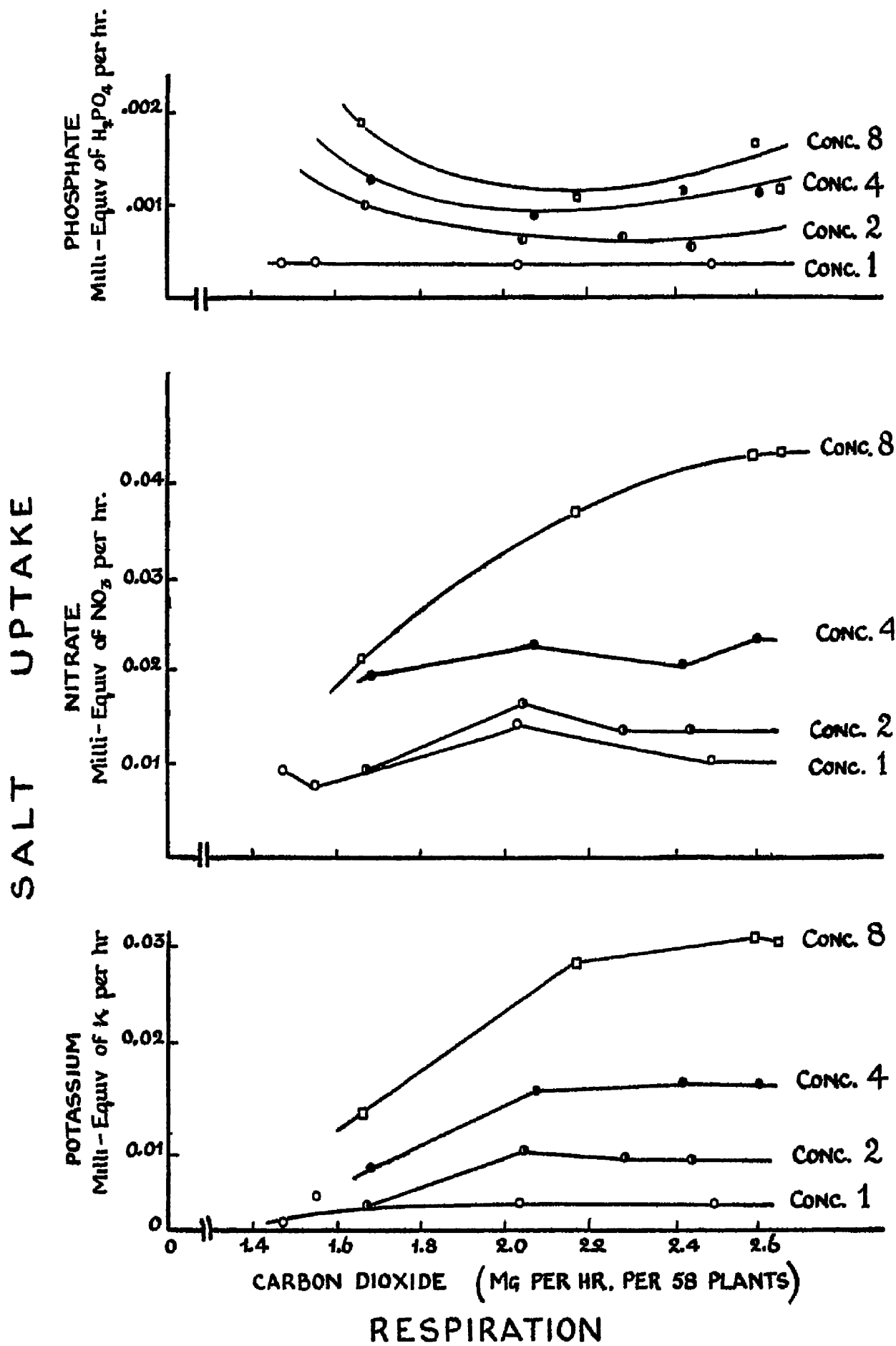


FIG. 10. Relation between respiration and salt uptake at different levels of nutrient concentration.

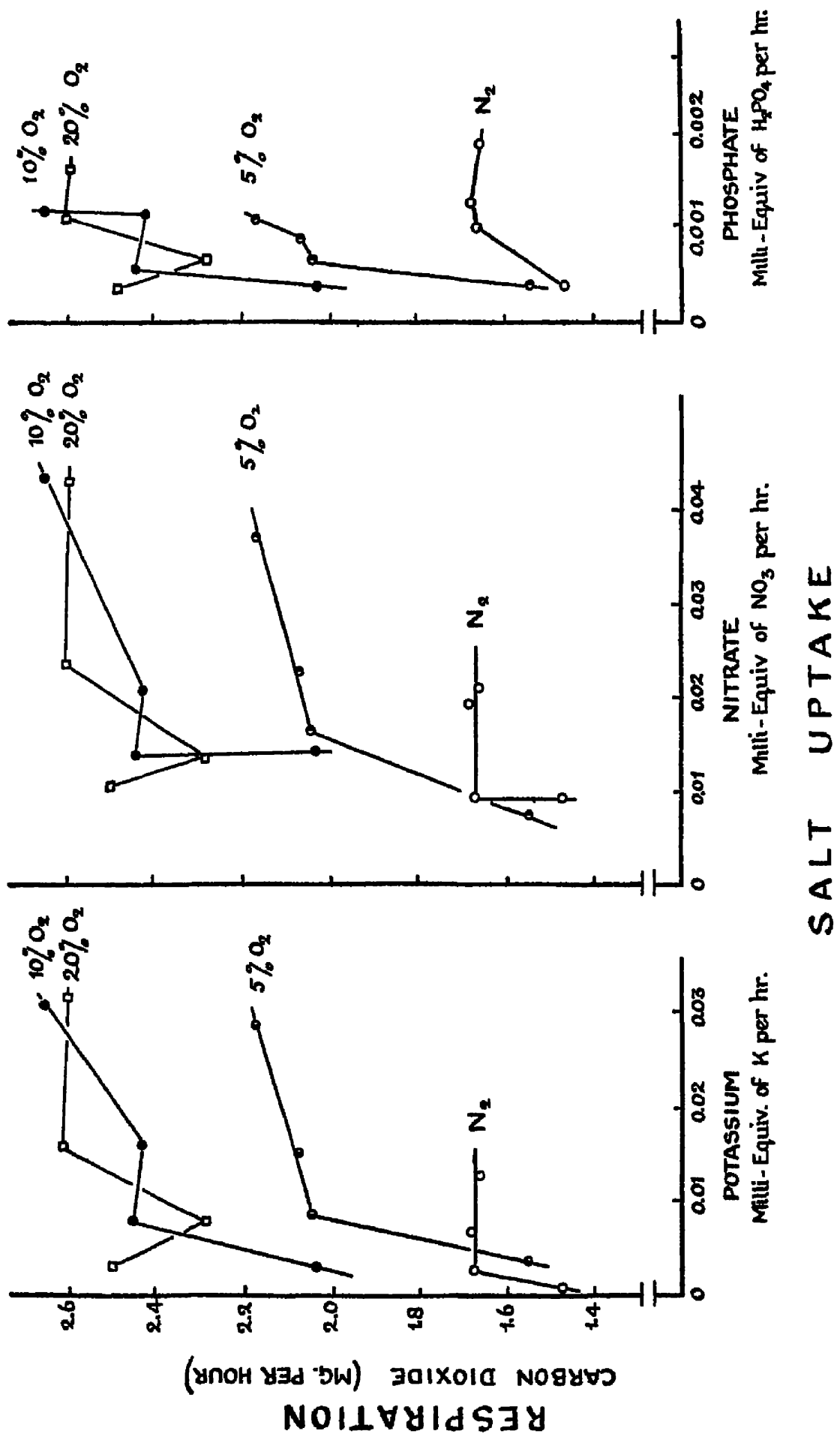


FIG. 11. Relation between respiration and salt uptake at different oxygen tensions.

that uptake per unit change in respiration falls to zero at all concentration levels. If, therefore, the uptake is dependent upon some process associated with respiration the efficiency of this process falls to zero long before uptake has reached its maximum value, as is seen from the three-dimensional diagrams in Figs. 7-9, where the curves of uptake with concentration all over the surface are still rising at the highest concentration level used.

The data can be presented in a different way as in Fig. 11, in which milligrams of carbon dioxide per hour are plotted against milli-equivalents of the ions taken up, and separate curves are given for the various oxygen tensions. For each ion the same relationship is seen, most clearly in the case of potassium. Over a short range of uptake a very large increase in respiration was found, but higher uptake rates tend to be independent of respiration rate. These curves could thus be represented by a series of 'limiting factor' curves with a steep limb at low rates of uptake and horizontal limbs, of constant carbon dioxide production, whose levels correspond with the various oxygen tensions in the nutrient solution. It would thus appear that for each oxygen tension a respiration rate corresponds and that the rate of uptake becomes independent of respiration rate. The respiration rate, therefore, is in the main independent of the nutrient uptake and is determined by oxygen supply only. Referring back to Table VIa, it is there seen that the only large effect on respiration is occasioned by a change from concentration 1 to 2, and that this increase is sufficiently large to make the nutrient concentration effect on respiration significant. These values in Table VIa are the mean for all the ions concerned, and it is now seen that this increase in carbon dioxide is associated with an increase in uptake of each of the ions studied.

Relations between respiration and uptake can be expressed as correlation coefficients between these factors and partial correlation coefficients eliminating the variables oxygen tension and nutrient concentration. The results are given in Table X, below.

TABLE X  
*Correlation of Respiration and Uptake*

	Total correlation.	Partial correlation eliminating nutrient concentration.	Partial correlation eliminating oxygen tension.
Nitrate . .	+0.401	+0.914*	+0.852
Phosphate . .	+0.073	-0.462	+0.946*
Potassium . .	+0.053	+0.857	+0.870

$P = 0.05$  for  $r = 0.878$ .

The results show that the total correlation between respiration and uptake is always negligible, but that the partial coefficients for all ions are high and positive when the effect of oxygen is eliminated, while a low negative value is found for phosphate when nutrient concentration is eliminated. Only two of the partial correlations are statistically significant. These figures merely confirm the conclusion that both oxygen tension and nutrient concentration are concerned with uptake of the ions.

## DISCUSSION

The apparatus described in this paper was devised to ensure the sterility of the culture medium during the course of the experiment, to maintain a predetermined oxygen tension around the roots, and to avoid diffusion effects in the solution by keeping the culture medium well stirred. In practice the results indicate that these ends were achieved. In this work for the first time a factorial experiment has been carried out in which the effects of oxygen tension and nutrient concentration have been explored simultaneously over a wide range and thus a study of the interaction of factors has been possible.

The plant material used was standardized and the external factors of light and temperature were maintained within reasonably constant limits, but even so variation among the batches of plants used, both in development and function, was considerable, and even for units of fifty-eight plants four replications were not sufficient to reduce the standard error of the experiment to less than the following values: Dry weight—roots 7.8 per cent., shoots 14.3 per cent.; respiration 18.3 per cent.; nitrate 19.7 per cent.; phosphate 15.3 per cent.; potassium 3.1 per cent. In this respect the high variability of the material, though grown under standard conditions, confirmed the experiences of Lundegardh (1945) with wheat plants.

It is unfortunate that some of the necessary data for a full investigation of the factors controlling salt uptake were not collected. The circumstances made this impossible. Thus the concentrations of the salts in the cell-sap of the plants are not known and therefore the degree of accumulation of the ions studied cannot be assessed. No data on the carbohydrate content of the roots are available. On this point the only information is that given by one experiment in which the roots were removed and the respiration and nitrate uptake compared with the roots of whole plants under similar conditions. The data in Table IV show that during the first 5 hours after excision of the shoots the respiration fell by only 2.5 per cent. and after the next 17½ hours had decreased by 69 per cent. The nitrate uptake, on the other hand, fell during the first 5 hours by 36 per cent. and after a further 17½ hours by 87 per cent. It is clear, therefore, that failure of uptake preceded reduction in respiration to a marked degree. Whether this decline in respiration and nitrate uptake was due to exhaustion of carbohydrate or to other causes such as a failure of oxygen supply to the roots cannot be decided from this experiment. The interpretation of the experimental results, therefore, is severely restricted in this work and it is not possible to compare them directly with those obtained by Hoagland and his colleagues working with excised roots or with those of Steward and his school using disks of storage tissues. It will be possible to discuss only the direct relations of uptake and of respiration, and nothing can be contributed to the deeper analyses of metabolic factors in the root.

In certain respects the data here presented are at variance with those given by Hoagland and Broyer (1936) and Steward, Berry, and Broyer (1936).

They are of the opinion that the optimal oxygen level for respiration and absorption are coincident, whereas in these experiments this is not the case. So far as carbon dioxide production is concerned the figures in Table VIa show that the maximum respiration occurs at 20 per cent. oxygen, the highest oxygen tension used, and the absence of interaction between oxygen tension and nutrient concentration indicates that the mean values given in that table will adequately represent the relation for all the nutrient concentrations. Little change in respiration occurs above 10 per cent. oxygen. This oxygen tension in the aerating gas corresponds with 2.4 ml. oxygen per litre. This value agrees with the results obtained by Hoagland and Steward, who also found maximum respiration with an aerating gas mixture containing 10 per cent. oxygen, though the actual concentration of the oxygen in the solution was not given. So far as salt uptake is concerned the results shown in Figs. 7-9 indicate clearly that the optimal oxygen tension varies as between the ions considered and the concentration of the nutrient solutions. For all ions, nitrate, phosphate, and potassium, there is little effect of oxygen tension on uptake at the lowest concentration, whereas at the highest concentration uptake is approaching a maximum value in 20 per cent. oxygen for nitrate and potassium, and phosphate has a minimum at 5 per cent. and no evidence of a maximum having been reached at 20 per cent. oxygen. That these shifts in the maximal uptake are real is indicated by the significant interaction which has been established for potassium.

The best proof that this work affords of the interaction of oxygen tension and nutrient concentration is that shown by the growth experiments in which barley plants were grown for 7 and 12 days respectively, aerated with oxygen in a relative nutrient concentration of 2 as against aeration with nitrogen in a relative nutrient concentration of 8. These results are given in Table III and show clearly that similar growth was made in the two cases. Although no analyses of the plants were made it is reasonable to assume that uptake of nitrate as the controlling factor was also similar.

Hoagland (1944) states that under anaerobic conditions no evidence of accumulation of any ion against a concentration gradient could be found in his experiments. In this work the salt concentration in the sap was not measured so that no data for accumulation can be presented. Nevertheless, uptake under anaerobic conditions was quite active for ions studied, indeed for phosphate uptake was as high in nitrogen as in 20 per cent. oxygen. Hoagland, however, worked with excised root systems and not whole plants as is the case here. It is very probable that under the conditions employed in these experiments the roots were not strictly anaerobic. Evidence that oxygen can be transported to the root from aerial portions of illuminated plants has been given by other investigators such as Cannon (1925, 1932), Cerighelli (1920), Raalte (1940), Vlamis and Davis (1944), Brown (1947).

Turning now to the relation between uptake and respiration, Lundegardh (1937, 1945) has postulated a basal respiration rate independent of ion absorption, and an anion respiration with a characteristic proportionality factor

relating rate of absorption to extra carbon dioxide production specific for each anion. In his view this respiration supplies the energy necessary for absorption against a concentration gradient, or alternatively for 'activating' diffusion into the cell when accumulation does not occur. This coefficient factor, he states, is not a constant, as Hoagland and Steward (1939, 1940) supposed, but is itself a variable, being influenced by the migration velocity of anions and cations. The value of this coefficient for the interpretation of the experimental results therefore loses much of its utility.

In view of the variability of the respiration rates in various experiments it would require a much more comprehensive body of data, performed in a single planned experiment, to test the significance of the departure of actual from predicted values than Lundegardh has presented. Lundegardh has in fact shown that respiration in salt solution is higher than in distilled water, which is in agreement with all other work including the present. The source of this extra carbon dioxide is not the major interest of Lundegardh. Steward and Preston (1941), on the other hand, with potato disks have carried out very extensive investigations on this point, and have shown that the extra carbon dioxide produced under these conditions is related to protein synthesis and originates probably from organic acid respiration; and Hoagland and Broyer (1936) and Steward and Preston (1941) also question the primary role of anions in controlling this metabolism. Indeed, in the view of Steward and Preston it is the cations that are mainly responsible and specifically so. In so far as this problem is concerned the work here reported can do no more than add some empirical information. The data given in Fig. 11 showing the relation between carbon dioxide production and uptake in milliequivalents of the three ions indicate, first, that over a very limited range of uptake large increases of carbon dioxide occur—but this holds for anions as well as cations—and second, that at the highest oxygen tension this rise does not occur in the case of nitrate and potassium ions. This is not inconsistent with Lundegardh's view that the anion effect may be primary and that the entry of the cation is dependent upon the anion uptake. It is, however, evident that over the main range of uptake, in the case of nitrate and potassium ions, the increasing uptake is quite independent of increased carbon dioxide production, the latter being a function merely of oxygen tension in the culture solution. These facts are at variance with the views of both Lundegardh and Steward, but data are lacking for a further analysis.

The data could be represented as limiting factor curves with a steep limb relating uptake rate with carbon dioxide production. Interpretation of such a limiting factor curve is entirely speculative. The simplest interpretation would be along the following lines. Entry of the ions is determined by combination at the cell surface with some constituent of the cytoplasm followed by an 'activated diffusion' through the cytoplasm similar to that established for conduction in the phloem, the rate of this process being dependent upon oxygen. The concentration of ions in the external solution will determine, by free diffusion from the solution, the rate of the first part of the process,

namely, the supply of ions available for transport across the cytoplasm. It is necessary also to postulate an increase in respiration with increasing supply of ions by direct effect upon the protein metabolism of the cell ('salt effect'). With increasing concentration of the solution the supply of ions by diffusion increases in proportion to the external concentration. At a given oxygen tension increase in external concentration, due to the more rapid arrival of the ions at the cell surface, leads to further increase in carbon dioxide production, but this rise reaches a maximum value determined by oxygen supply so that the curve relating carbon dioxide production to external concentration is unaffected by a concentration at relative levels higher than 2. This is shown very clearly in the mean values of Table VIa, where an increase in the external concentration from 1 to 2 increases carbon dioxide production considerably, but above this concentration oxygen supply becomes the limiting factor.

The uptake rate of the ions is everywhere related to the external concentration. In Figs. 7-9 it is seen that for potassium, over the whole range of nutrient concentration, the uptake is proportional to the concentration; the contours of potassium uptake rate parallel with the nutrient concentration axis are all linear. For the other ions a linear relation does not hold, but nevertheless uptake continues to rise up to the highest level of concentration. It is quite evident that concentration over the range studied is the chief factor determining uptake. Returning now to Fig. 11, the rising limb of the curves is due to the increased carbon dioxide production due to the 'salt effect' on metabolism accompanied by an increase in uptake due to the concentration effect. This holds over the range of relative concentration 1 and 2. Above this at higher concentrations uptake continues to increase with nutrient concentration, but carbon dioxide production is now limited by oxygen supply and the curves become horizontal, indicating the independence of carbon dioxide production and uptake, the various oxygen tensions thus giving, over this part of the range, horizontal limbs at various heights. If this interpretation is correct the rising limbs should all extrapolate back to the respiration rate in distilled water. The curves in Fig. 11 indicate that this may be so except for the values at the highest oxygen tension. The data presented in Fig. 3 show that the respiration rate in distilled water, at the highest oxygen tension, is 30 per cent. below that in a solution of relative concentration 4. There must therefore also be a rising limb of the curve in 20 per cent. oxygen in Fig. 11. Reference to Table VIa shows that the value for respiration at nutrient concentration 2 and oxygen tension *D* (20 per cent.  $O_2$ ) is quite evidently low, so that the curve for this oxygen tension in Fig. 11 is inconsistent with the rest.

The interaction between oxygen tension and nutrient concentration as shown in Fig. 7 can now be elucidated. At the lower nutrient level the rate of arrival of the ions at the cell surface is so slow that 5 per cent. oxygen activates diffusion to a rate sufficiently high to ensure transport at a maximum rate consistent with the concentration, whereas at the highest nutrient

concentration a somewhat higher level of oxygen tension is necessary to maintain a sufficient transport of the ions. For phosphate a different state of affairs is indicated. Here uptake is lowest with 5 per cent. oxygen and the explanation as given above fails. Qualitative differences between the different ions evidently exist. To compare directly the uptake rate of the various ions account must be taken of the varying relative concentrations of the ions in the nutrient solution used. To do this the uptake has been calculated in terms of milli-equivalents taken up as a percentage of the milli-equivalents present in the solution at the start of an experiment. These values for the various ions for the sixteen treatments are given in Table XI below.

TABLE XI  
*Relative Uptake: Milli-equivalents of NO<sub>3</sub><sup>-</sup>, H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, and K<sup>+</sup> per hour as Percentage of Milli-equivalents in Solution at the Start of Experiment (58 plants)*

Relative concentration.		Oxygen tension.				
		Ion.	A (0 %).	B (5 %).	C (10 %).	D (20 %).
8	{	NO <sub>3</sub> <sup>-</sup>	0.228	0.404	0.482	0.476
		H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>	0.626	0.360	0.384	0.546
		K <sup>+</sup>	0.748	1.93	1.80	1.82
4	{	NO <sub>3</sub> <sup>-</sup>	0.416	0.538	0.449	0.553
		H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>	0.874	0.596	0.782	0.742
		K <sup>+</sup>	0.746	1.70	1.81	1.90
2	{	NO <sub>3</sub> <sup>-</sup>	0.400	0.711	0.599	0.586
		H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>	1.19	0.808	0.754	0.834
		K <sup>+</sup>	0.512	1.92	1.68	1.75
1	{	NO <sub>3</sub> <sup>-</sup>	0.794	0.684	1.22	0.871
		H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>	1.02	1.01	0.986	1.01
		K <sup>+</sup>	0.454	1.63	1.31	1.31
Mean	{	NO <sub>3</sub> <sup>-</sup>	0.459	0.584	0.687	0.622
		H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>	0.927	0.694	0.726	0.783
		K <sup>+</sup>	0.615	1.80	1.65	1.70

Mean total: NO<sub>3</sub><sup>-</sup> = 0.588; H<sub>2</sub>PO<sub>4</sub><sup>-</sup> = 0.782; K<sup>+</sup> = 1.44.

The very small reduction in the percentage concentration of the ions in the solution during the experiment (max. 1.9 per cent. per hr.) indicates that there was sufficient excess in volume of solution used not to interfere with uptake by depletion. The mean values over all treatments show that the relative rates of uptake of NO<sub>3</sub><sup>-</sup>, H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, and K<sup>+</sup> were as 1:1.3:2.4. These figures represent the mean relative permeabilities of the cytoplasm to these ions. From these figures also the efficiency of uptake for the various nutrient concentrations can be assessed. It is seen that for potassium an increase in the relative concentration from 1 to 8 leads to a rise in relative uptake from 1.18 to 1.57, whereas for both anions there is a fall over the same range from 1.01 to 0.48 for phosphate and 0.89 to 0.40 for nitrate. The interpretation of these results is not clear. So far as the anions are concerned it might be attributed

to accumulation at the higher external concentration resulting in a falling gradient across the cytoplasm, but even this supposition would not account for the effect seen with potassium. Nor for the same reason would any postulated permeability changes due to the change in salt concentration account for the results. This work indicates the following relations between salt uptake, concentration, oxygen supply and respiration. Respiration rate is affected by nutrient concentration ('salt effect'), for in every case rise in concentration from level 1 to 2 is accompanied by a large increase in  $\text{CO}_2$  production. At a concentration above level 2 salt uptake rises with concentration and is independent of respiration (Fig. 11). The level of respiration is determined by oxygen supply. From anaerobic conditions to 5% oxygen salt uptake rises very markedly but above 5% oxygen tension has little effect. Clearly  $\text{O}_2$  tension affects salt uptake, but whether this is due to energy supplied by respiration, 'activated diffusion', or some other metabolic effect cannot be decided from these experiments.

Uptake of phosphate differs from that of the other ions in that the rate in anaerobic conditions is as great as that in 20%  $\text{O}_2$  with a minimum uptake in 5%  $\text{O}_2$ .

In these experiments a balanced culture solution has been used whose composition is the same as that employed in previous nutrition studies in this laboratory, and there seems no good reason to complicate the issue in salt uptake experiments by using solutions of single salts, for the advantages of simplicity in the composition of the external solution are more than offset by the injurious effects of single salts on root growth.

The advantage of the factorial design, so well realized in agricultural experimentation, has been shown to hold for the laboratory experiment also, and only by its use can a sufficiently wide range of conditions be explored and interactions be established so as to make a deeper analysis of the problems of salt uptake profitable. The application of factorial methods to these complex situations and the type of mathematical analysis necessary to unravel the effects of single ions has been briefly discussed by Richards (1944).

The work reported in this paper is of a preliminary nature and has been published to indicate the possibilities of the apparatus described. The bearing of these results on the general problem of salt uptake has been indicated, and they are not without importance with regard to cognate problems such as the growth of plants when conditions around the roots are anaerobic. The finding that growth under anaerobic conditions can be maintained at a level as high as that with full aeration, by merely increasing the nutrient concentration, may be of some practical importance, for it appears to show that the poor growth in badly aerated soils may to a large extent be due to a starvation of the primary nutrients consequent upon a slower uptake for a given concentration of soil nutrients. The evidence for transport of oxygen to the root system found by other investigators also receives some support from this work.

## SUMMARY

An apparatus is described for the estimation of root respiration and ion uptake in seedlings. Respiration is determined by measuring the  $\text{CO}_2$  liberated from the roots growing in a circulating culture solution maintained at the desired temperature and oxygen tension, and kept aseptic. To minimize the complication due to root growth the experimental period was reduced to 5 hours.

Barley seedlings grown under standard conditions were used throughout.

Isolation of the roots caused an immediate decrease of nitrate absorption followed  $5\frac{1}{2}$  hours later by a rapid decline in respiration. Nitrate uptake fell during the first 5 hours by 36 per cent., in the next  $17\frac{1}{2}$  hours by 87 per cent.; corresponding respiration changes were 2.5 and 69 per cent.

By the use of a factorial design of experiment the interaction of oxygen tension and nutrient concentration on ion uptake and respiration of the roots was studied over a wide field ( $\text{O}_2$  tension 0–20 per cent.; nutrient concentration 3.2–24.5 milliatmos.).

Increase in oxygen tension in the culture solution led to an increase in ion absorption and in respiration.

The effect of oxygen tension on the mean rate of uptake varies with the different ions studied. With nitrate and potassium a large increase in uptake occurs when oxygen tension is increased from 0 to 5 per cent.; further increase up to 20 per cent.  $\text{O}_2$  has little or no effect upon uptake. With phosphate uptake is minimal at 5 per cent.  $\text{O}_2$  and is as great in total absence as in presence of 20 per cent.  $\text{O}_2$  in the culture solution (Figs. 7–9).

The effect of oxygen tension in increasing the mean respiration rate of the roots is seen over the whole range (Table VIa).

Significant interactions of the factors were established. The effect of oxygen tension on the absorption of ions is dependent upon the concentration of the ions; it is very small at the lowest concentration, and increases with every rise in concentration. The oxygen tension for maximal absorption is not the same as that required for maximal respiration. The details for the individual ions are shown as three-dimensional graphs in Figs. 7–9.

The effect of increased absorption with increasing concentration of ions is found at all levels of oxygen tension, and is equally marked in the anaerobic solution. For nitrate and phosphate the uptake is relatively less as concentration increases; with potassium ion uptake is proportional to concentration over the whole range of concentrations used. The interaction of oxygen tension and ion uptake appears as varying slopes of the contours drawn on the solid models at varying oxygen levels (Figs. 7–9).

It was shown that barley seedlings could grow for some time as well in anaerobic as in aerated solutions so long as the concentration of the ions was sufficiently raised (Table III, Fig. 4).

It is concluded from the relation of ion uptake to respiration rate at various oxygen levels (Fig. 11) that over a very limited range of concentration of ions

increase in ion absorption is accompanied by increased  $\text{CO}_2$  production, but a steady rate of  $\text{CO}_2$  production is reached, the level being dependent upon  $\text{O}_2$  supply; by further raising the concentration of the ions uptake goes on increasing without any corresponding change in respiration. This is true of anion ( $\text{NO}_3^-$ ) and kation ( $\text{K}^+$ ). The rise in respiration with ion concentration is attributed to a 'salt effect', the effect of  $\text{O}_2$  tension is due directly to  $\text{O}_2$  supply. Concentration changes directly affect ion uptake and indirectly  $\text{CO}_2$  production so that over part of the range studied a correlation appears between uptake rate and respiration. The behaviour of phosphate is to some extent different from nitrate and potassium, particularly in the high rate of absorption in anaerobic solution (Figs. 8, 10). The bearing of these results on current theories of absorption of ions is briefly discussed.

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# Experimental and Analytical Studies of Pteridophytes

## XIII. On the Shoot Apex in a Tree Fern, *Cyathea Manniana* Hooker

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With Plates VIII and IX and sixteen Figures in the Text

### INTRODUCTION

IN these studies fern apices have been used extensively in the investigation of morphogenetic processes, partly because the apical meristem of the adult shoot is both of considerable size and distinctive histological constitution, partly because there is no complication of secondary thickening in the developing shoot, and not least because, as experience has shown, the fern apex will remain viable after a considerable amount of experimental manipulation, provided the apical cell is not damaged. The ferns include the largest living pteridophytes, the Cyatheaceae and Dicksoniae being represented by arborescent species with stout trunks which may grow to a height of 20 ft. or more. Such species may occupy a conspicuous and important place in the plant associations in which they occur. A consideration of the growth, development, and organization of these large forms suggests many interesting problems for investigation. While the general morphology and anatomy of *Cyathea*, *Dicksonia*, and related genera have been investigated (Ogura, 1938, where the literature is cited in detail) there has been little exploration of developmental processes, particularly in the larger forms. During a visit to the Belgian Congo in 1946 the writer collected the shoot apices of a number of well-grown specimens of *Cyathea Manniana* Hooker, together with the apices of lateral rhizomes. The nature of the apical meristem, the formation and arrangement of leaf primordia and buds, and the organization of the differentiating tissue systems in these materials are described and discussed in this paper.

The organization, during the individual development, of the apical meristem in ferns of small or intermediate stature such as *Dryopteris aristata* or *Matteuccia struthiopteris* has already been studied (Wardlaw, 1943, 1943a, 1944). In broad essentials it was found that the shoot apex changes little throughout development from the young sporophyte to the large adult; there is, of course, an increase in the size of the apical cone. But what is the state of affairs at the apex of a well-grown tree fern? Little or no attention has apparently been given to this problem in the relevant literature (Bower, 1889, 1923; Schuepp, 1926; Ogura, 1938). We may therefore inquire if a study of the apices of tree ferns throws any new light on the organization of

the fern plant in general, and whether the attainment of large size is attended by significant changes in the formative region, i.e. the shoot apex, or in the positions of organs, or the distribution and configuration of the several tissue systems.

#### MATERIALS AND METHODS

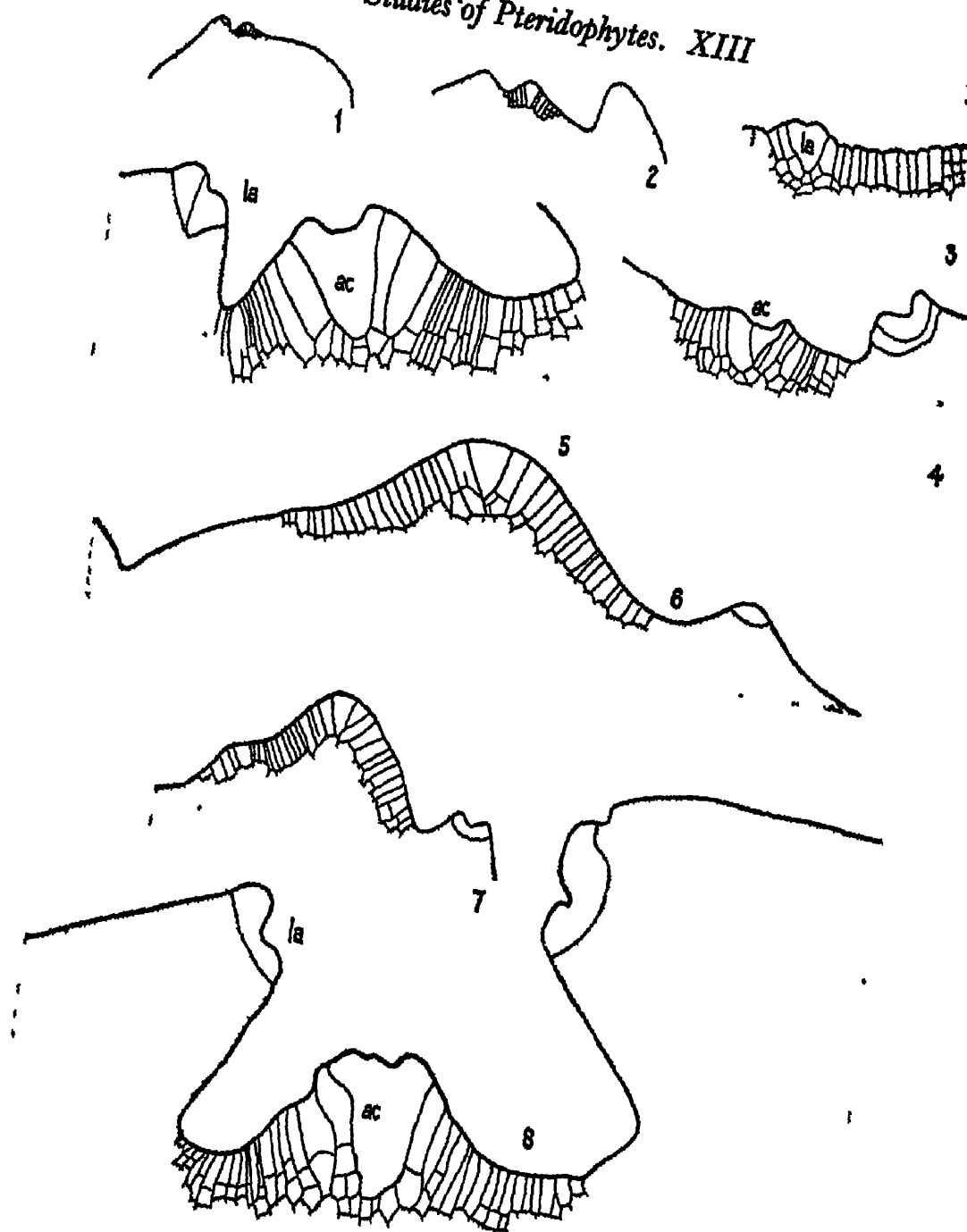
Apices of large specimens of *Cyathea Manniana* some 12 ft. high, and of lateral rhizomes of different sizes, were preserved in 70 per cent. ethyl alcohol, embedded in wax, and cut transversely and longitudinally. As young sporophyte plants of *C. Manniana* were not available, those of *C. dealbata* have been used to complete the ontogenetic series.

For purposes of comparison reference has been made to the apices of large plants of *Dryopteris aristata* Druce and *Matteuccia struthiopteris* Todara, and to those of the oil palm (*Elaeis guineensis*) among flowering plants.

#### SHOOT APEX OF LARGE TREE FERN

It may be said at once that, with the exception of some features to be mentioned below, the apices of the largest specimens which the writer has been able to examine are closely comparable with those of leptosperangiate ferns of smaller stature, e.g. *Dryopteris aristata*. Thus the *apical meristem*, as defined by Wardlaw (1943), consists of a single superficial layer of prism-shaped cells, of distinctive appearance and staining reaction, these cells originating by the division of the tetrahedral ('3-sided') apical cell and by further anticlinal divisions.

A notable feature is the very large size of the apical cell and of the prism-shaped cells to which it gives rise. The apical meristem of a large *Cyathea* plant is, in fact, like the magnified replica of a smaller apex (Text-figs. 1-8 and Pl. VIII, Figs. 1-4). The apices illustrated in Pl. VIII, Figs. 3 and 4, are closely comparable in size and appearance. But Fig. 3 is the apex of a young sporophyte magnified 128 times, whereas Fig. 4 is the apex of a large tree fern magnified only 17 times, i.e. the apex in Fig. 4 is 7.5 times the size of that in Fig. 3. Thus the organization of the apical meristem apparently persists without major change from the young sporophyte to the adult plant, the principal change with increasing size being an increase in the size of the individual cells of the meristem. If the apex of a lateral rhizome of *C. Manniana* (Text-fig. 4) is compared with the apex of a large shoot of *Dryopteris aristata* (Text-fig. 6), it will be seen that while the cells of the apical meristem are of approximately equal size, the latter apex is considerably more extensive than the former. Indeed, the surface area of the apical meristem of *D. aristata* in Text-fig. 6 is closely comparable with that of the largest *Cyathea* apex examined, Text-fig. 8. As will be seen below, the latter is very closely beset and enclosed by the developing leaf primordia. It is probable that apical cells larger than those illustrated in Text-figs. 5 and 8 and Pl. VIII, Figs. 1, 2, will be found in the larger tree ferns; meanwhile these are probably the largest apical cells so far figured in the literature. By way of contrast, the writer

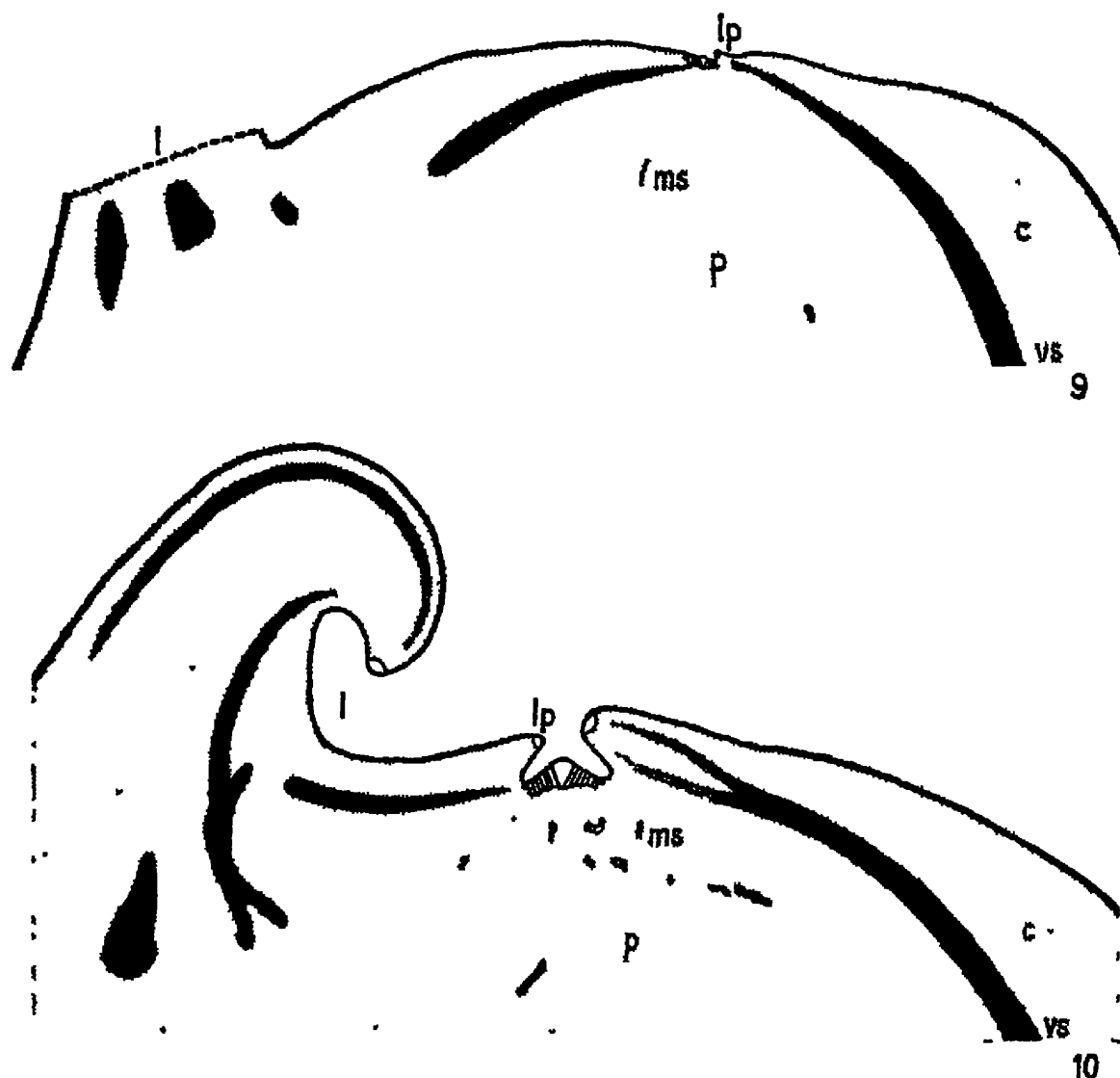


TEXT-FIGS. 1, 2, 3, 4, 5, 8, apices of *Cyathea* spp ; Fig. 6, *Dryopteris aristata*; and Fig. 7, *Matteuccia Struthiopteris*, in longitudinal median section. Figs. 1, 2, apices of young sporophytes of *C. dealbata*; Fig. 3, initial cell of leaf of *C. Manniana*, Fig. 4, apex of rhizome of *C. Manniana*; Figs. 5, 8, large apices of erect shoots of *C. Manniana*. *ac*, apical cell, *la*, leaf apex. ( $\times 50$ )

has also figured, in transverse and longitudinal section, the terminal apices of well-grown oil palms (*Elaeis guineensis*), Pl. VIII, Figs. 6, 7. These show how very different is the cellular organization in the two types of apex. As in other leptosporangiate ferns, the appearance of a new leaf primordium in *Cyathea* is indicated by the conspicuous enlargement of one of the prism-shaped meristematic cells (Text-fig. 3; Pl. VIII, Figs. 8, 9). During their

further development leaf primordia are characterized by very large and conspicuous apical cells.

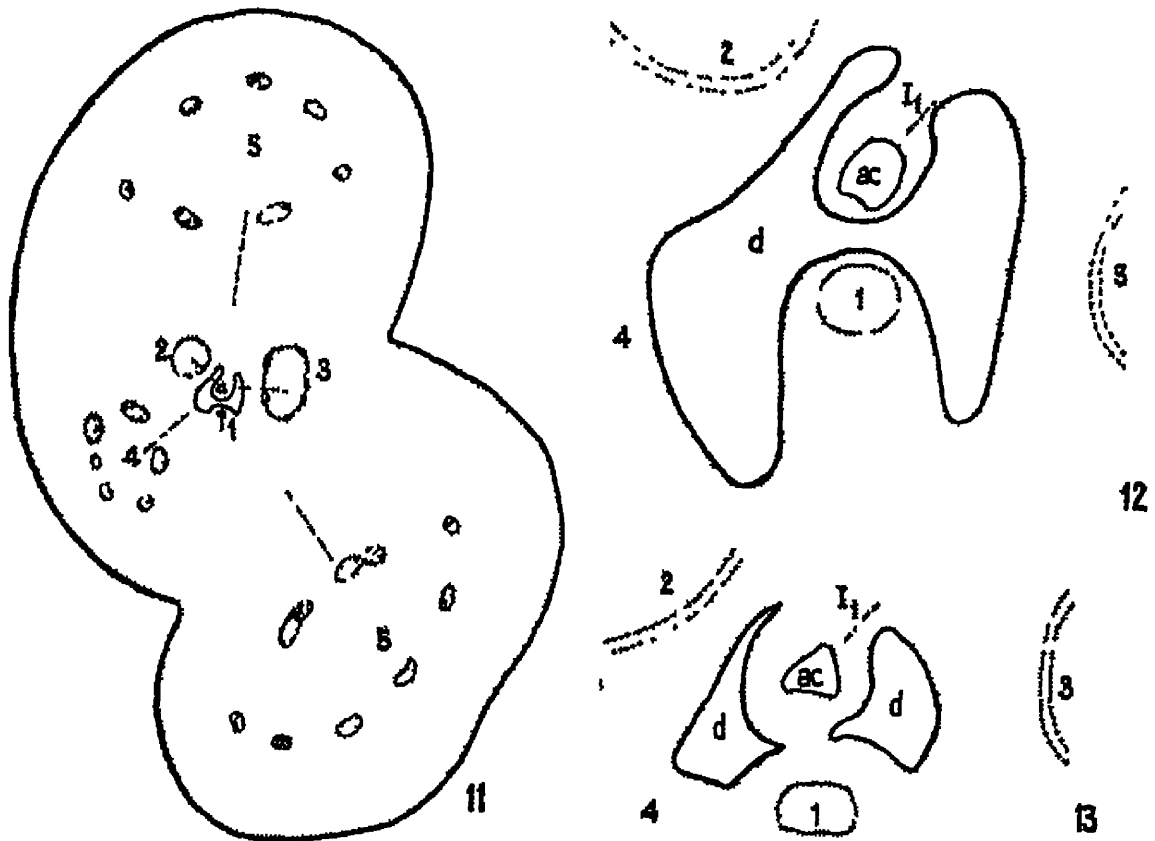
If, now, we consider some general features of the distal region of the main shoot or of a stout rhizome, Text-figs. 9, 10, the apical meristem is seen to be of small size relative to the tissue systems (cortex, stele, and pith) to which it gives rise. This conspicuous increase in size in the sub-apical



TEXT-FIGS. 9, 10. *Cyathea Mammiana*: longitudinal median section of the apex of a stout rhizome, Fig. 9, and of a large erect shoot, Fig. 10. *l*, leaf; *lp*, leaf primordium; *p*, pith; *c*, cortex; *vs*, vascular strand; *ms*, medullary strand. ( $\times 8.5$ .)

region is largely due to the extensive development of parenchyma in cortex and pith—a state of affairs also evident in ferns of smaller stature (Wardlaw, 1945). Even in rhizomatous shoots where the leaves are small and persist chiefly as scale leaves with little laminate development, the leaf-bases become conspicuously distended (Text-fig. 11). In erect shoots the leaf-bases grow to large size whilst still in proximity to the shoot apex: as a result the apical cone occupies the depression formed between them (Text-figs. 8, 10; Pl. IX, Figs. 4, 5, 10, 11). Apical cones seated in depressions are also known in other ferns, e.g. *Osmunda* and *Marattia*. These sunken growing-points in *Cyathea* are in marked contrast to the freely projecting ones found

in *Dryopteris* and *Matteuccia* (Text-figs. 6 and 7). The relative smallness of the apex and the massive development in the sub-apical region are very evident in serial transverse sections (Pl. VIII, Fig. 5), the apex being situated in an irregular saucer-like depression (also Pl. IX, Figs. 10, 11). In the course of development the leaf-trace, initially a small strand of incipient vascular tissue, enlarges to a cylinder with a central pith; on further growth it becomes disrupted into a number of widely separated meristeles.



TEXT-FIGS. 11-13. *Cyathea Mannana*: transverse sections of the apex of a small rhizome. Fig. 11, general view showing the apical cell, the adjacent tissues, and six leaf primordia. The broken lines indicate angles of  $138^\circ$  between successive primordia. ( $\times 8.5$ .) Figs. 12, 13, sections at two levels, showing how the depression surrounding the apical cone has been pulled out tangentially by the enlargement of the leaf-bases. 1, 2, 3, leaf primordia, in order of increasing age;  $I_1$ , position of next primordium to be formed; ac, apical cell; d, depression. ( $\times 50$ .)

#### LEAF FORMATION AND PHYLLOTAXIS

The details of leaf formation are as in other leptosporangiate ferns: from an early stage the development of each new primordium proceeds from the division of a conspicuous 'two-sided' apical cell (Pl. IX, Figs. 8, 9, Text-fig. 3). In point of fact, the details of leaf formation in the ferns need to be reconsidered: this will be the subject of a later paper in this series.

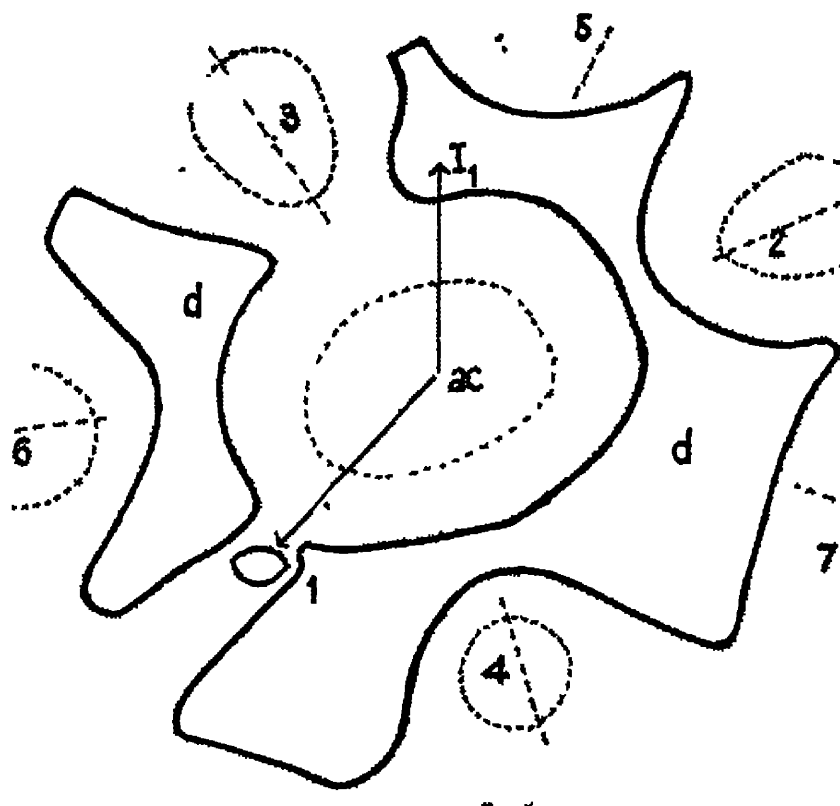
Text-fig. 11 shows the apex of a small rhizome in transverse section with six leaf primordia disposed on a right-handed spiral, the angle of divergence being approximately  $138^\circ$ . This angle has been indicated in the illustration: it will be seen that the arrangement of primordia is very regular indeed. But in large apices the distribution of primordia may be considerably less regular.

Text-figs. 14 and 15 and Pl. VIII, Fig. 5, illustrate sections through a large apex at different levels. The angles between primordia were as follows: 1 and 2,  $156^{\circ}$ ; 2 and 3,  $104^{\circ}$ ; 3 and 4,  $160^{\circ}$ ; 4 and 5,  $137^{\circ}$ ; 5 and 6,  $125^{\circ}$ ; 6 and 7,  $156^{\circ}$ . The next primordium to be formed,  $I_1$ , will arise between leaves 2 and 3. Text-fig. 16 and Pl. IX, Fig. 11, by contrast, show a large apex in which the angles between the last-formed primordia approximate closely to  $138^{\circ}$ .

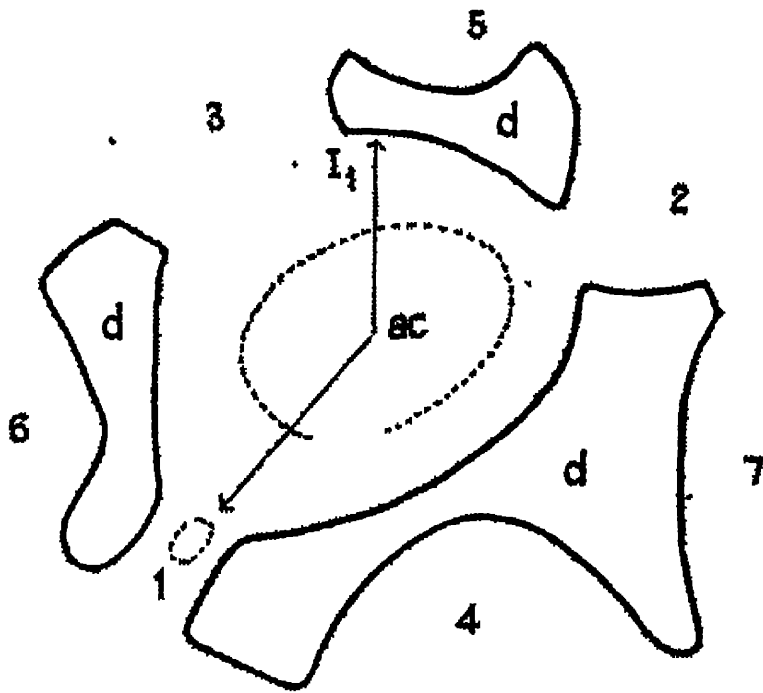
Considerable interest attaches to the *shape*, in transverse section, of the depression in which the apical cone is situated. This depression comprises the several small depressions lying between adjacent primordia and between these primordia and the apical cone. They vary considerably in shape, with the result that the depression surrounding the apex is of rather irregular outline. The notable feature is the extent to which each interfoliar depression becomes distended in the tangential direction. This is undoubtedly due to the very considerable tangential enlargement of the radially adjacent leaf-base. These distended depressions are in close proximity to the apical meristem. Hence it would appear that, as each leaf primordium develops, that region of the apical meristem which lies above its axil is subjected to tensile stress (Wardlaw, 1947, 1948). The outlines of the depressions illustrated in Text-figs. 12–16 and Pl. VIII, Fig. 5, and Pl. IX, Figs. 10, 11, strongly support this view. These illustrations suggest that stress will be developed in the apical meristem by the primordia (leaves 1–7) surrounding it and in close proximity to it. The stress set up by any primordium will diminish as it recedes from the apex during growth, e.g. leaves 7, 8, 9, 10, &c. The illustrations also show that the next primordium to be formed ( $I_1$ ) will appear in that region of the apical meristem in which tensile stress is minimal. The region of minimal tensile stress is also the largest unoccupied area of the apical meristem, above the last-formed primordium, as described by Hofmeister (1868) and by M. and R. Snow (1947, where their earlier investigations are reviewed). Text-figs. 14 and 15 illustrate the further point that the apical growing-point is elliptical rather than circular in cross-section, a further indication of the effect on the apex of the tangentially enlarging leaf-bases. As indicated in the preceding section, the shoot apex in the largest specimens examined was rather smaller than might have been expected. The apex, indeed, has the appearance of being restricted by the rapid enlargement of the basal regions of the surrounding primordia.

#### LATERAL BUDS

As other investigators have shown, lateral buds in tree ferns may be of occasional or frequent occurrence (Scott, 1874; Schoute, 1906; Bower, 1912, 1913; Ogura, 1927). In *C. Manniana*, as observed by the writer, lateral buds are of occasional occurrence. They may develop into horizontal rhizomes of considerable thickness (2–3 cm.) and length (several metres). Eventually some of these rhizomes develop as vertical shoots, thus giving rise to the typical arborescent form. Occasional examples of very young buds in



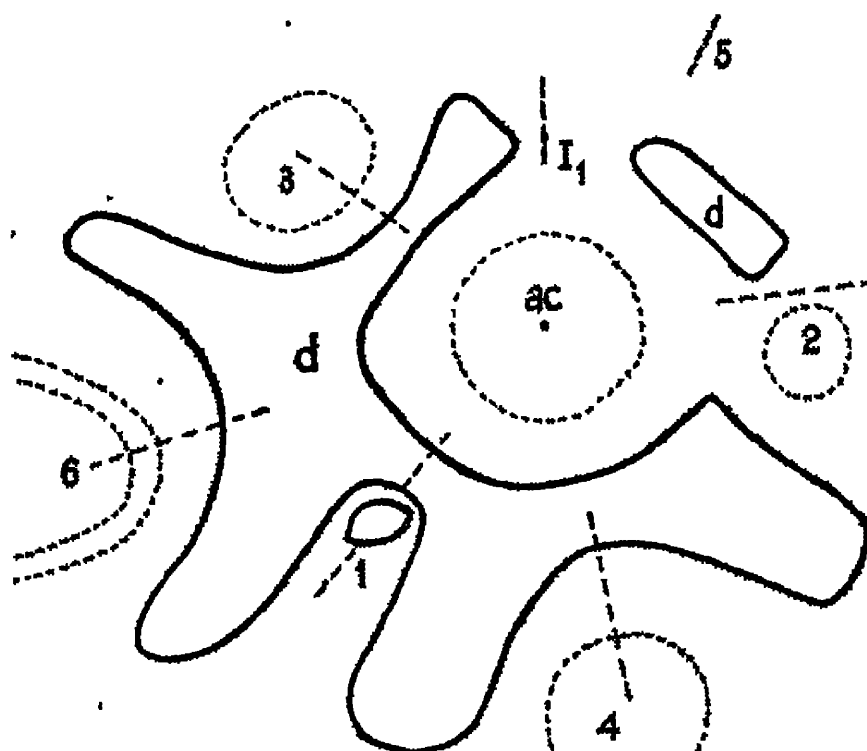
14



15

TEXT-FIGS. 14, 15. *Cyathea Manniana*: transverse sections at two levels of the apex of a large erect shoot. These sections show how the depression surrounding the apex has been pulled out tangentially by the enlargement of the leaf-bases. *ac*, apical cone; *d*, depression; 1, 2, 3, &c., leaf primordia in order of increasing age; the apical cell of the youngest primordium, 1, is shown; the incipient vascular tissue is indicated by broken lines; the broken radial lines through the medium plane of each primordia were used in measuring the angles of divergence cited in the text; *I*<sub>1</sub>, position of next primordium to be formed. (× 45.)

proximity to the shoot apex have been observed (Pl. IX, Figs. 12, 13). These arise laterally on a leaf-base, as in *Dryopteris aristata*. The relation of the bud-stele to the shoot-stele (in those instances where the former is conjoined with the latter) is as in *Dryopteris*. Notwithstanding its eventual position on a swollen leaf-base, a bud rudiment initially occupies a position which may either be described as axillary or interfoliar (Wardlaw, 1943a).



TEXT-FIG. 16. *Cyathea Manniana*: transverse section of the apex of a large erect shoot. General description as in Text-figs. 14, 15. The apical cell of the youngest primordium, 1, is shown. The next primordium,  $I_1$ , will be formed in the region of minimal tangential tensile stress. The broken radial lines indicate what the position of the several primordia would be if the angle of divergence were consistently  $138^\circ$ , i.e. the primordia occur with a high degree of regularity. ( $\times 45$ .)

#### VASCULAR ANATOMY

In *C. Manniana* numerous anastomosing fine strands are present in the pith and may end blindly there. The appearance of the dictyostele and leaf-trace and the origin and distribution of the accessory strands are as described by Ogura (1938) for other species.

#### DISCUSSION

From an early stage in the individual development the shoot apex in leptosporangiate ferns is characterized by a well-defined organization. It might perhaps be thought that as the plant increases in size, with the attendant obconical shoot development and enlargement of the apical growing-point, structural changes might take place in the latter exemplifying the size-

structure correlation (Bower, 1930, 1948). For example, there might be a limit to the size of the single apical cell; and when that limit was reached a change in the organization of the apical meristem would take place. In the present investigation it has been shown that during development from the small sporophyte a few millimetres in length to the adult plant 3–4 metres in height, the apical growing-point does enlarge considerably. The extent of this enlargement—about 7–10-fold—is clearly indicated in the illustrations. But none of the changes with increase in size suggested above has in fact been realized: in appearance, outline, formative activity, arrangement of parts, growth, and differentiation, the large apex of an adult shoot is closely comparable with the small apex of a young sporophyte, the former being an enlarged replica of the latter. In both, the sides of the apical cone diverge at an angle of approximately  $90^\circ$ . In its morphogenetic activities as in its structural organization the apex thus remains singularly unchanged throughout the ontogenetic development, notwithstanding the vast increase in the size of the plant and in the number of its component parts. The formation of the lateral organs and the differentiation of tissues, both of which are determined at the growing-point, are thus independent of the actual size of the meristem. It can scarcely be doubted that there are some differences in the physiological processes, or in the rates at which they take place, in cells of large size as compared with those of small size. Furthermore, it seems probable that there are differences in the magnitude of the physical forces at work in a large as compared with a small apex. Nevertheless, the configuration of the meristem, the formation and distribution of lateral primordia, and the cellular pattern persist with only slight modifications from the juvenile to the adult state. The major differences can in fact be related to developments in the sub-apical region. It may therefore be inferred that the organization of the apical meristem is of such a nature that a high degree of stability is maintained over a considerable range in size.

In some ferns one initial cell is present at the shoot apex, in others several initial cells are present, and in yet others a fluctuating intermediate condition is found (Bower, 1923). In the group of ferns distinguished by Goebel (1880–1) as *leptosporangiate*, growth at the apex proceeds from the division of a single apical cell; in *eusporangiate* ferns growth proceeds from a group of three or four apical initials. These differences extend also to development in leaves, roots, and the spore-producing members. The leptosporangiate type has been described by Bower (1891) as being based on a less massive and more precise plan of segmentation, and the eusporangiate type on a more massive and less precise plan of construction. Within the coherent and naturally related group of the ferns, then, we have apparently two different plans of construction, linked by intermediate types. From the evolutionary standpoint the eusporangiate type is held to be the more primitive condition (Bower, 1891). Sachs (1887), in working towards a causal explanation of development at the apex, considered that the cellular pattern there is determined by the bulk of the organ and has no other significance. But Bower

(1923) has summarized evidence which indicates that the relation between bulk, or massiveness, and apical segmentation is in no sense simple and direct: the manner of segmentation is not, in fact, directly dependent on actual size. This view is supported by the new data presented here, for it has been shown that the very large apices of *Cyathea Manniana*, though in every sense massive or bulky, retain both their leptosporangiate character and the organization established in the young sporophyte. From these facts it may be inferred either that genetic (hereditary) factors are of paramount importance, or, as indicated above, that the shoot apex as a structural system has great stability and is functionally efficient over a considerable size range.

Sections are illustrated which show how markedly different is the apex of *Cyathea* in cellular character from that of a flowering plant—the oil palm. (The apices of many other flowering plants might equally well have been figured.) Although there are great and evident differences between the apex of a large leptosporangiate fern and the vegetative apices of flowering plants (themselves exemplifying a considerable histological diversity), nevertheless the principal results of apical activity are broadly comparable in the two groups. Each gives rise to the shoot type of organization, i.e. to a vasculated axis bearing lateral members in a regular sequence and, in some instances, with a strictly comparable spatial distribution. Many ferns and flowering plants, for example, have spirally arranged leaves with an angle of divergence of approximately  $138\frac{1}{2}^{\circ}$  (the so-called 5/13 phyllotaxis). It is almost impossible to avoid the conclusion that the two examples of shoot formation are similar because the same physical and physiological factors are at work in both. Yet how unlike is the cellular organization in the two apices. Indeed, the contrast seems to afford further support for de Bary's aphorism that 'the plant forms (or fashions) cells, not cells the plant'. Bower (1923) has summarized the position for the ferns by saying that the genesis both of external organs and internal tissues is independent of segmentation (although at times the two may coincide) and that apical segmentation and 'morphological definition', whether external or internal, are distinct processes, each of which is determined by the apical region as a whole and not by its segments.

Conspicuous and important as the apical cell in a tree fern is, the growing-point functions as a whole. Wilson (1925) has said that 'the physiological anatomy of the individual cell falls into the background . . . and the apparently composite character which the multicellular organism may exhibit is owing to a secondary distribution of its energies among local centres of action'. Earlier, both Hofmeister and Sachs (who regarded the apical cell as a gap in the plan of construction and without physiological significance) had maintained that in plants organ growth is the primary fact and cell formation only of secondary significance, while Whitman (1893), supporting this view, pointed out that 'for the same purpose', e.g. the formation of an organ, one, several, or many cells may be used. The truth of this statement becomes apparent when the apices to the different classes of vascular plants are compared. Yet this matter may require and merit further consideration. If the

apical cell of a leptosporangiate fern shoot be destroyed, the growth of the shoot ceases, though growth will probably be continued by one of its lateral buds; and if the apical cell, which becomes evident in the leaf primordium at a very early stage, be injured, the primordium ceases to grow. Thus while it may be maintained that the apex functions as a whole, the apical cell is an indispensable component and is essential to its harmonious and orderly activity, i.e. its 'normal' development. This argument of course applies only to shoots in which growth proceeds from a single apical initial cell.

Apical growth, with closely comparable results, can proceed from a meristem of many small cells, none of which is of distinctive size or appearance. As Ball (1946, 1947) has shown, some of these apices can be split into four parts by two vertical incisions at right angles, and all four parts are capable of developing into separate shoots. This is not possible in leptosporangiate ferns, though the development of lateral buds in an incised shoot takes place readily (Wardlaw, 1947). But in the leptosporangiate fern shoot, large or small, the apical cell must remain intact, or shoot growth will not take place.

It may be that in plants of a particular genetical constitution the differentiation of a large and distinctive apical cell is a necessary result of forces present in the growing-point; but what these forces are and how they act is still obscure. Such an explanation, if valid, would support the view that the plant or organ determines the tissue pattern, the formation of an apical cell being a characteristic result of the particular forces acting in the fern apex; or in the words of D'Arcy Thompson (1942), the growing-point might be regarded as 'a comprehensive field of force . . . somehow shaping the whole organism, independently of the number, magnitude and form of the individual cells', the formation of the apical cell being a particular and necessary expression of this force in certain plants. It thus appears that the relationship between the apical cell in ferns and the apex as a whole is, in a sense, reciprocal; for if the existence of the apical cell depends on the configuration of the apex, the continued development of the apex depends on the activity of the apical cell—a conclusion not unlike that reached by investigators such as Schleiden and Schwann more than one hundred years ago.

Important differences exist between the large apices of tree ferns and those of dicotyledonous trees: in the former, as we have seen, the growing-point is (microscopically) a large and conspicuous region, the bulky shoot to which it gives rise being the result of primary activity alone; in the latter the growing-point is minute, the thick shoot which develops being the result of secondary thickening. In arborescent monocotyledons such as the oil palm the growing-point is also small, but behind it there develops a massive shoot without the action of a cambium. These interesting contrasts, to which Bower (1948) has called attention, merit further study.

Development from the young sporophyte to the adult state is marked by a 7- to 10-fold increase in the linear dimensions of the apical meristem; but the increase in the diameter of the shoot is approximately 100-fold (i.e. from 1 mm. to about 10 cm.). This great increase in the diameter of the shoot is

largely due to the development of parenchymatous tissue in the cortex and pith, a tendency also evident in other ferns (Wardlaw, 1945). With the enlargement of cortex and pith, changes take place in the cross-sectional pattern of the shoot; but these changes relate to developments in the sub-apical region of the shoot, not to those in the formative region, i.e. the apical meristem.

The larger apices of *Cyathea* occupy a slightly sunken position. This is due to the considerable enlargement of the bases of the leaf primordia surrounding the apex. The shape of this apical depression is of interest in the study of phyllotaxis, particularly in relation to factors which may determine the position of new primordia. As each new primordium develops, its basal region undergoes a marked enlargement, particularly in the tangential direction. This affects the shape of the depression, i.e. it becomes pulled out tangentially. The observations which have been made, and the illustrations in the text, leave little doubt that while developing primordia are in close proximity to the apical meristem, the latter is subject to tangential tensile stress above the axil of each primordium; the next primordium to be formed develops in that region of the meristem in which tensile stress is minimal. Experimental evidence has already been obtained which supports these views (Wardlaw, 1947a, 1948).

Ogura (1927) has considered the question of phyllotaxis in different tree ferns, his observations being based on the leaf-scars on the trunk. He concludes that a phyllotaxis of  $2/5$  is replaced by one of  $3/8$  as the plant increases in size and that a phyllotaxis represented by higher members of this series, presumably  $5/13$ , may be observed in still larger plants. The writer's observations on leaf arrangement at the apex show that an angle of divergence of approximately  $138.5^\circ$  ( $= 5/13$ ) is present in small rhizomes and in stout shoots. In some instances the angle of divergence between leaf primordia is very constant; in others it may vary considerably.

Ogura (1927) has also described the presence of lateral buds in a number of different species of tree fern. In some of these he observed that the bud-stele became conjoined with the shoot-stele; in others no vascular connexion was established. Buds showing the latter condition are regarded by Ogura as being adventitious. In the materials of *Cyathea Manniana* under consideration, buds of different ages have been investigated. These buds closely resemble those of *Dryopteris* in that they are typically associated with the leaf-bases: there is, indeed, no reason to regard them as being different from those of *Dryopteris* in any important respect. Whether the bud-stele becomes conjoined with the shoot-stele or not depends on the time of development of the bud rudiment (Wardlaw, 1943a). In the present writer's view the adventitious buds of Ogura are, therefore, merely bud rudiments that have developed on an older region of the shoot.

#### SUMMARY

An account is given of the apical meristem in the tree fern, *Cyathea Manniana* Hooker, comparisons being made with the smaller apices of

rhizomes and young sporophytic plants, and with the apices of other ferns and flowering plants.

In the largest specimen examined the apex has the normal organization of leptosporangiate ferns, i.e. the large apex is a magnified replica of the small apex of a young plant. The inference is drawn that the organization of the apical meristem in ferns is such as to possess stability and functional efficiency over a considerable size range.

The remarkable increase in the girth of the shoot and leaf-bases is largely due to the increase in parenchymatous tissue in cortex and pith.

In large specimens the apex occupies a slight depression formed by the swollen bases of the adjacent leaf primordia. The shape of this depression is variable; this is due to the very considerable tangential enlargement of the leaf-bases. The indications are that the apical meristem is subjected to tangential stress above the axil of each primordium, the next primordium to be formed being situated in the region of minimal stress.

Phyllotaxis as observed at the shoot apex may be regular or irregular, an angle of divergence between primordia of approximately  $138^\circ$  ( $= 5/13$  phyllotaxis) being common both in small rhizomes and large shoot apices.

Lateral buds, which are associated with the leaf-bases, bear the same relation to the shoot and shoot-stele as those of other leptosporangiate ferns.

Some general features of the apices of vascular plants are discussed.

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## EXPLANATION OF PLATES VIII AND IX

Illustrating Professor C W Wardlaw's paper on *Cyathea*

All figures are from untouched photographs

## PLATE VIII

Figs 1, 2 *Cyathea Manniana* Apex of large erect shoot in transverse and longitudinal median section, showing the very large apical cell ( $\times 128$ )

Fig 3 *Cyathea dealbata* Apex of young sporophyte in longitudinal section ( $\times 128$ )

Fig 4 *Cyathea Manniana* Large shoot apex, i.e. that illustrated in Fig 2 ( $\times 17$ ) (The apices in Figs 3 and 4 are generally comparable, but note the difference in magnification)

Fig 5 *Cyathea Manniana* Large apex in transverse section, showing the apical cell, the irregular depression in which the apical cone is situated, a very young leaf primordium (below the apical cell), older leaf primordia, and the incipient vascular tissue surrounded by developing parenchymatous tissue ( $\times 115$ )

Figs 6 and 7 *Elaeis guineensis* Shoot apices in transverse and longitudinal section ( $\times 128$ ), i.e. same magnification as the apices in Figs 1 and 2

## PLATE IX

Figs 8-13 *Cyathea Manniana*

Figs 8, 9 Apical cell of young leaf primordium in transverse and longitudinal section at a very early stage of development ( $\times 200$ )

Fig 10 Transverse section of the apex of a small rhizome, showing the apical cell and adjacent tissues, and the elongated depression round the apical cone ( $\times 45$ )

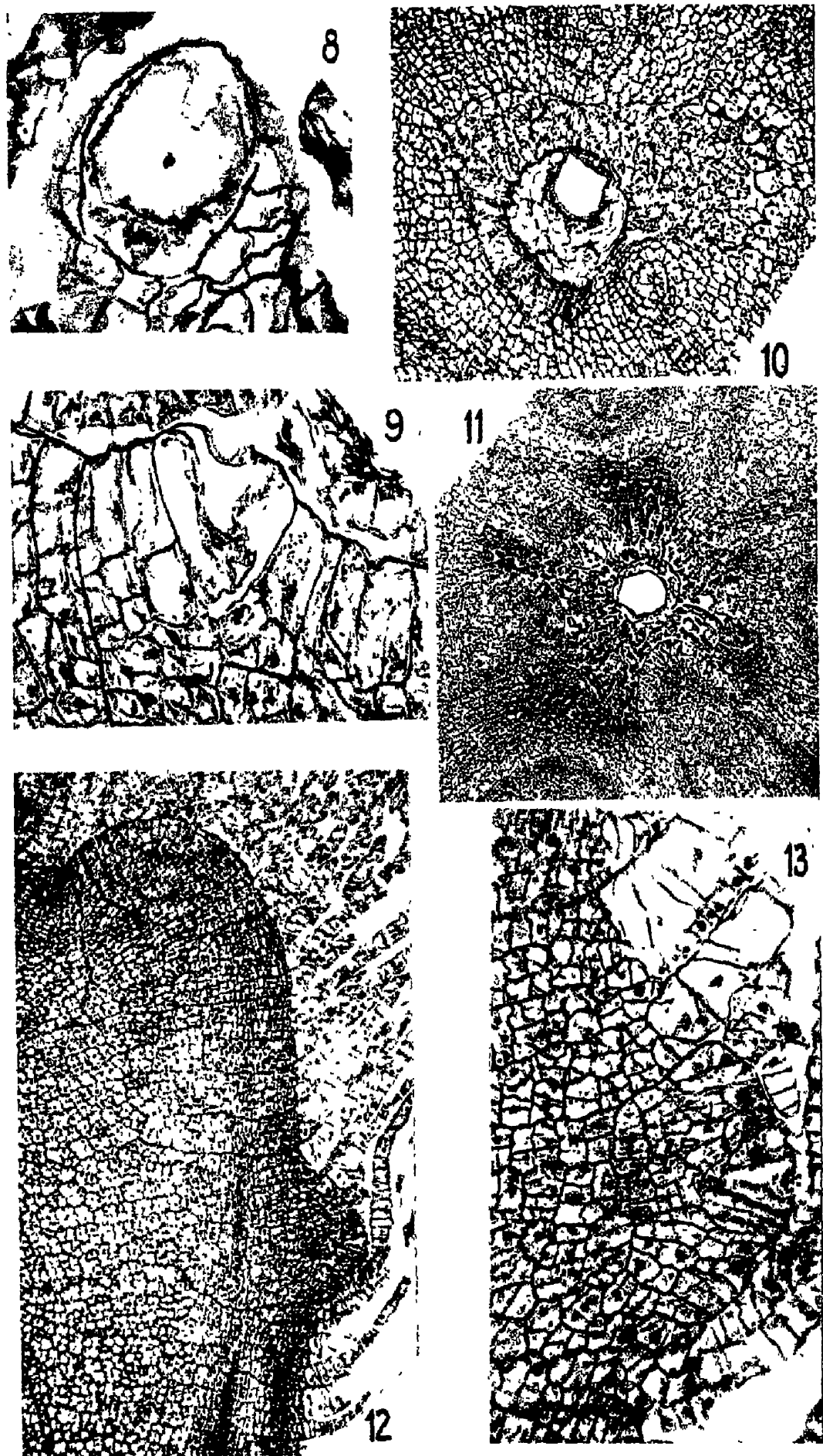
Fig 11 Transverse section of the apex of a large erect shoot, showing the apical cell, the irregular depression surrounding the apex, and several young leaf primordia ( $\times 18$ )

Fig 12 Longitudinal section through the lateral flank of a leaf-base, showing a very young bud. ( $\times 45$ .)

Fig 13. As in Fig. 12: the apical cell, which has divided, is clearly seen ( $\times 135$ )



WARDLAW—CYATHEA MANNIANA



WARDLAW—CYATHEA MANNIANA

# Structure of the Testa and its relation to Germination in the *Papilionaceae* Tribes *Trifoliae* and *Loteae*

BY

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With four Figures in the Text

## INTRODUCTION

**A**LTHOUGH the majority of seeds of most plants begin to absorb water when they are placed in damp surroundings at a temperature suitable for germination, a very high percentage of the seeds of some plants remain unchanged. Harrington (1916) states that seeds of this kind occur in members of *Cannaceae*, *Liliaceae* (*Convalariaceae*), *Leguminosae*, *Geraniaceae*, *Malvaceae*, *Solanaceae*, *Convolvulaceae*, and many observers (Ewart, 1908; Guppy, 1912; Rees, 1911) agree that this condition is especially characteristic of the seeds of *Leguminosae*. A seed which does not absorb water freely and swell when placed under conditions favourable for germination is said to be hard or impermeable. In each sample of most species of leguminous seeds which have been tested at this time there were both permeable and impermeable seeds in various proportions. Guppy (1912), in his comprehensive study of germination, has shown that the weight of an impermeable seed was not affected by any changes in the hygrometric state of the atmosphere or by prolonged immersion in water. The weight of a permeable seed was stable, being subject to hygroscopic variation, and permeable leguminous seeds were shown to double their weight when immersed in water. By baring permeable and impermeable seeds of their coats he made an impermeable seed react as a permeable one and, by absorbing water from the air, gain 11 to 12 per cent. in weight in a very few days. The increase was maintained, but in a diminishing degree, until stability was reached with the increase reduced to 3 or 4 per cent. The permeable seed preserved an average weight but displayed a greater hygroscopic variation than when the coat was on. By puncturing or filing the seed coat he obtained similar but more highly modified results. If the seed coats were removed from the seeds and treated separately, the coats from the impermeable and permeable seeds were affected by the hygrometric state of the atmosphere in the same manner as were the corresponding permeable and impermeable seeds. It is quite probable, however, that after the coats are removed water is transmitted through the inner surface of the seed coat. Considering Ewart's (1908) and Crocker's (1916) work, Guppy thinks that the most practical tests for the potential vitality of an impermeable seed are to be found in its constant weight under all ordinary conditions and

over a period of years. Continued hygroscopic reaction brings about molecular change, terminating in loss of germinating power, so that hygroscopicity limits the life of permeable seeds, and its absence in impermeable seeds ensures longevity. Seed impermeability and seed longevity are therefore related. Longevity, according to Ewart (1908), is an hereditary property inherent in the protoplasm of certain seeds and developed by natural selection as an adaptation to the special conditions of life. He reports a germination percentage of 22 for 18-year-old seed of *Melilotus albus*, but in old seeds of many other leguminous species the percentage of germination is extremely low. Nine out of forty-five species of leguminous seeds retain germinating power after 14 years' storage in dry air (De Candolle, 1846), and eighteen out of ninety species after 25 to 80 years' storage (Ewart, 1908). It is generally agreed, however (Coe and Martin, 1920; Ewart, 1908; Hambly, 1932; Rees, 1911), that the cause of impermeability is to be found in the incapacity of the seed coat to transmit water and oxygen freely, but there is considerable disagreement as to the particular layers of the seed coat that are impermeable.

#### STRUCTURE OF A LEGUMINOUS TESTA

The testa of *Lotus Requiemii* shows most of the structural characteristics of the seed coat of the common species of Loteae and Trifoliae. Although this general structure (Fig. 1) is modified in some species, the testa of that plant will be described as typical of those members of these tribes which have been investigated and it will be followed by discussions of variation in other species.

On the outer surface there is a cuticle (*a*) of medium thickness. Directly beneath the cuticle the Malpighian cells (*e*) form a closely packed palisade-like layer, each member of which is more or less entangled with its neighbour. An individual Malpighian cell is somewhat club-shaped in form with the wider end towards the inside of the seed. The outer end is pointed and embedded in the sub-cuticular layer (*b*). These Malpighian cells are marked transversely about one-quarter of the way in by a light line (*c*) which appears to separate the subcuticular layer from the inner part (*d*) of the Malpighian cell. The light line is indistinct and the cell walls and cellular contents may be seen through it. The Malpighian cells are underlain by a layer of empty cells (*f*) which are noticeably regular in dimensions and arrangement. This layer may be conveniently termed the intermediary layer. The osteosclereid cells (*g*) are spool-shaped and separated by large intercellular spaces. The nutrient or pigment layer (*h*) is two to three cells in depth and is composed of cells of various sizes. A band (*i*), probably made up of collapsed nutrient cells, is present just below the nutrient layer. The aleurone layer (*j*) breaks away very easily during the preparation of sections, but usually enough remains (as in the figure) to show that this consists of at least a row of uniform angular cells.

There is marked variation in the structural features of some of the other species of Trifoliae and Loteae. Usually the cells are very small and closely compacted near the hilum, but they are larger and much more uniform on the

opposite side of the seed. This, together with the fact that the seeds of all the species of the tribes in question are different in size and shape, shows that comparative measurements of the cells of the testa are of little significance. It is even difficult to choose the exactly corresponding part of each testa.

There is only a slight difference in the thickness of the cuticle on seed of different species. During the preparation of the sections the cuticle is often broken away completely or separated in part from the underlying cells.

In the sub-cuticular portion the tips of the Malpighian cells often take the form of distinct caps which are united at their base and reach out through a bedding matrix towards the cuticle. The Malpighian cells occur in all species and are always set perpendicularly to the cuticle. They vary from species to species in their longitudinal and transverse dimensions and in their density of packing.

The light line has been examined only by ordinary diffused light, yet it appears to differ in thickness and luminosity from one species to another, being readily visible or quite indistinct. Although very little is known about its function or nature, it is one of the most interesting features. In some leguminous seeds Pammel (1899) identified two or three light lines, but usually he found only one. He also discovered a light line in the walls of an ovary and those of a sporangium, but he did not name the plants. He came to the general conclusion that a light line always occurs in a part of the structure of a reproductive body, and that it has some function whose nature remains undetermined. Pammel gave a thorough analysis of the literature bearing on the nature and function of the light line, and showed that previous investigators had reached no unanimity in their conclusions. He concluded that the light line differs in optical and chemical properties from other parts of the wall of the Malpighian cell. Hambly (1934) has recently made an examination of the light line in *Melilotus albus* under polarized light. By arranging his source of light so that it was first parallel then perpendicular to the axis of the Malpighian cells, he found that there were in reality two lines. He is of the opinion that the light line is a condition resulting from the juxtaposition of the suberin in the caps and cellulose in the lower portion of the Malpighian cells.

The inner portion is the most uniform part of the Malpighian cell in every species.

The intermediary layer is not present in all species. It may be a layer composed only of the outer portions of the spool-like osteosclereid cells.

In some of the species the osteosclereid cells look like sheaves of strands constricted transversely across the middle of the bundle and spreading out at each end to join the neighbouring sheaves. The osteosclereid cells are always present, but they are much larger and more conspicuous in some seeds than in others. In some seeds they are hour-glass shaped and separated by large triangular or small prismatic intercellular spaces. These spaces do not usually occur in the same plane and therefore do not appear to be regular in every section. The cells are filled with longitudinal strands, canals, or finger-like processes in some species, while in others the cells are opaque.

The size and number of cells in the nutrient layer vary considerably in each species. These cells are termed nutrient because in the growing seed, according to Pammel (1899), they conduct elaborated and unelaborated food products, collapsing as the seeds mature so that in time the cavity appears as a mere line.

The aleurone layer, which usually adheres to the nutrient layer in cutting, is an even row of regular cells that is always present in the unimpaired seed.

#### STRUCTURAL CAUSES OF IMPERMEABILITY

A number of investigators have endeavoured to establish a relation between impermeability and the structure of the testa. Most of the work has been done on some species of Leguminosae, particularly *Melilotus albus*. White (1908) is of the opinion that the cuticle prevents the penetration of water into the seeds of many species. She thinks that the cuticle develops as the seed ripens, and that its presence ensures a long life for the seed when it is stored in dry air. She shows that the thickening of the cuticle increases the degree of impermeability in the small- and medium-sized seeds. In the larger seeds she thinks that the Malpighian cells are more resistant. White's conclusions are supported by Rees (1911), who shows that seed coats of *Indigofera arrecta*, *Cytisus albus*, and *Acacia melanoxylon* are more impermeable because of the presence of a thicker cuticle.

Hambly (1932) found that the Malpighian caps, which are attached to one another at their base, prevented the entrance of water into the seeds of *Melilotus albus*. He treated the seeds with a fresh 1 per cent. solution of osmium tetroxide which gives a rapid penetration through any opening or permeable area and makes a black or dark brown coloration. In this way he determined which seeds being permeable were capable of absorbing water, and the exact area of penetration. Hambly found that the Malpighian caps formed a complete line of impermeable suberin. The only part of the impermeable seed which was affected by the osmium tetroxide was the hilar region, while the permeable seed showed blackening in other places as well. Rees (1911) agrees with Hambly in part when she says that it is probable that the outer membrane (sub-cuticular layer) acts as a cement substance to hold the cuticularized ends of the cells together and causes impermeability in *Melilotus albus*.

Coe and Martin (1920), working with *Melilotus officinalis* and *Melilotus albus*, maintain that the light line and not the sub-cuticular layer or Malpighian caps prevent the absorption of water. In the coats of the impermeable seeds the light line was usually broader, the Malpighian cells thickened more below the light line, and the main cavities of the Malpighian cells were more reduced and farther below the light line than in the coats of the permeable seeds. In many of the permeable seeds, but not in all, canals were found to extend across the light line.

Rees (1911) attributes impermeability in *Acacia lophantha* and *Canna indica* to cuticularized thickening in the Malpighian cells.

TO BREAK DOWN IMPERMEABILITY

The destruction of the seed coat to such an extent that it will become permeable to water is the main objective behind many methods which have been devised to hasten and improve the germination of impermeable seeds. Some methods are so efficient that they are used commercially, while others which give equal or even better results have not been put into such common use because they are too complicated or expensive. Chloroform, ether, sodium hydroxide, potassium nitrate, sulphuric acid, and many other reagents have been used in an attempt to increase permeability, some with success and others without. They may act either as a solvent for the chemical constituents of the seed coat or they may break down the structure and make the seed coat permeable. Crocker (1916) believes that seed coats are often of such a colloidal nature as to be modified by various concentrations of many reagents and thus give the embryo a chance to develop.

Sulphuric acid has given very good results and is the method in most common use. Prillwitz (1930), Hambly (1932), Rees (1911), and Coe and Martin (1920) have used sulphuric acid to treat leguminous seeds. Various concentrations and durations of treatments affected the germination in different ways. Prillwitz (1930) found that a fairly strong acid must be used, that the duration of the treatment must be short, and that the acid must be completely washed out before the seed is placed in conditions suitable for germination. He determined, for thirteen species, the duration of treatment which gave good germination at once and was followed by very little mortality when the seeds were subsequently stored for 3 weeks. Hambly (1932) was able, by the use of osmic acid, to see that the sulphuric acid had penetrated the seed coat sufficiently to macerate the sub-cuticular and Malpighian layers and thereby make a passageway for the water. Coe and Martin (1920) found that the action of sulphuric acid destroyed the cuticle, the sub-cuticular layer, and the caps in 5 minutes, but did not affect the light line after 15 minutes except in making the canals and pores visible. Verschaffelt (1912) has used alcohol and other organic liquids to induce water absorption in experimenting with some impermeable seeds from the sub-families Caesalpineae and Mimosaeae. Once the micropyle or clefts, which he found on the surface of some of the seeds, had been filled with alcohol, water was absorbed quite freely.

Various methods of temperature treatment have been used. Busse (1930) was able to increase germination of some leguminous seeds from 14 to 90 per cent. by freezing them in liquid air. He found that it did not decrease the viability and might even be beneficial in retarding the ageing of the seed. He thought that it stimulated the liberation and distribution of enzymes so that in some of his tests germination was not only increased but growth stimulated. It was probable that the tiny cracks which formed when the impermeable membrane was frozen brittle permitted the entrance of water and caused germination to be increased. In some cases extreme heat treatments give similar results. According to McNairn (1917), many farmers boil

*Medicago arabica* 1 minute to ensure good germination. He also had similar success with *Trifolium reflexum*, but with other leguminous seeds no success whatever. Chippindale (1933) worked with *Dactylis glomerata* and found that seed which had been previously soaked in water at any time and subsequently dried, had germination accelerated. McNairn combined the soaking in cold water for 12 hours with boiling for various lengths of time, getting best results for 1 minute boiling. Hambly (1932) used heat to make seeds permeable. Harrington (1916) found that freezing impermeable seeds when they were wet caused germination of some seeds if they had not been previously softened. Alternating temperatures of 10 or less degrees centigrade with 20 or more degrees centigrade caused the germination of many impermeable clover seeds. The effect was greatly increased by previously exposing the seeds to damp conditions at a temperature of 10° C., and greatly decreased by previously exposing the seeds to damp conditions at a temperature of 50° C.

High pressure has been shown to have a beneficial effect in decreasing the impermeability of seeds. As the duration of high pressure is increased, it has been shown by Davies (1928) that, as a general rule, there is an increase in the percentage of permeable seeds, and a decrease in the percentage of impermeable seeds; in every case the proportion of permeable seeds was higher and that of impermeable seeds lower than in the control. There was a variation in the duration of treatment required for various genera. *Medicago sativa* required a shorter exposure to a pressure of 2,000 atmospheres than did *Melilotus albus* to produce the same results. Rose (1915) has used what he terms a direct pressure blower through which seeds can be fed and blown against the points of a bank of needles. Rapidity of germination as well as total germination was shown to increase with the use of this machine. This rapidity of germination helped produce more vigour and make the crop more uniform in size and age.

The process of mechanical hulling may act as an abrasive agent and cause permeability. Hambly (1932) shows by his osmic acid treatment that the water is absorbed more freely when the seed coat is broken mechanically, and he believes that the swelling proceeds from the damaged areas, the embryo emerging there during a damaged or abnormal germination. Another treatment which Hambly found effective for *Melilotus albus* was to toss the seeds in a bottle. He suggested that the strophliar cells in impermeable seeds are in a state of metastable equilibrium which, when upset, produces permeability through a split along the middle lamellae, linearly arranged in the plane of symmetry. After he had used several types of impactors he concluded that light impacts were as effective as heavy, but that a sufficient number of impacts to ensure a strophliar contact was essential. When this treatment was done with sufficient force to upset the stability of the tissue, which was in a state of tension, a cleft appeared on the separation of the long, slender Malpighian cells permitting the passage of water through the now permeable seed coat.

#### METHOD OF APPROACH

It is evident from this outline of methods which have been employed to bring about permeability that the seed coat is often impermeable to water. The aim is therefore to describe the testas of representative genera of Papilionaceae, tribes Trifoliae and Loteae, and to explain the relationship of their structure to impermeability and hence poor germination. Several species have been described individually by former investigators, and Pammel (1899) has made a survey of the anatomical characters, of chiefly the genera in Gray's 'Manual', in which he describes five species which are being used at this time: *Melilotus albus*, *Melilotus officinalis*, *Medicago sativa*, *Medicago lupulina*, and *Trifolium pratense*, but no relation is established between the structure of each species and its impermeability.

Hambly (1932), Coe and Martin (1920), Rees (1911), and others have separated permeable and impermeable seeds within a species. Coe and Martin made a comparison between permeable and impermeable seeds. They found no difference whatever in the chemical structure, and made no coherent relation between the canals crossing the light line in all permeable seeds and in no impermeable seeds.

Guppy (1912) stated that impermeable seeds might be distinguished from permeable seeds by external differences in certain plants as *Entada polystachya* and *Axyris amaranthoides*. This was not true, according to Harrington (1916), in any plants which he examined, nor has it been found true in the species of Loteae and Trifoliae which have been examined in the present investigation.

#### Material

Seeds were all collected from the Royal Botanic Gardens, Kew, and the University Botanic Garden, Cambridge, England. All seeds were carefully cleaned by hand to prevent any scarification which might be caused by mechanical cleaning. The seeds which were used for germination were in most cases those which had been completely ripened. A few were chosen, however, to see if they could be more easily sectioned before they had become too hard and yet were old enough to be properly matured. The seeds were collected as soon as they ripened and each lot of the several species was added to from time to time to obtain a uniform sample of the season's crop. To make sure that the longevity of the seeds had very little effect upon the percentage of germination, the seeds which were used for germination were usually not more than 12, and in no case more than 21, months old. One of the germination tests was made as soon as the seeds were collected and further tests were made at intervals throughout the period of the investigation to neutralize the effect which might have been produced if a period of after-ripening were required by any of the leguminous seeds in question.

#### Methods

For each germination test, 100 seeds were germinated on blotting-paper under aseptic conditions. For sectioning, the material was embedded in

paraffin. Great difficulty was experienced in cutting the extremely hard seeds on a rotary microtome. A few seeds were collected from the plafits as soon as they were properly matured and yet were soft enough to cut more easily. They

TABLE I

*An Average of the Results obtained in Four Separate Germination Tests, each of Thirty-five Days' Duration*

Species arranged in order of merit of germination.	Number in percentage		
	Germinated or permeable.	Mouldy.	Ungerminated or impermeable.
<i>Trifolium hybridum</i> . . .	98·0	1·0	1·0
<i>Anthyllis tetraphylla</i> . . .	88·0	12·0	0·0
<i>Trigonella caerulea</i> . . .	81·0	2·0	17·0
<i>Medicago cuneata</i> . . .	69·0	16·0	15·0
<i>Medicago lupulina</i> . . .	62·0	1·0	37·0
<i>Melilotus officinalis</i> . . .	62·0	6·0	32·0
<i>Trifolium pratense</i> . . .	60·5	10·0	29·5
<i>Dorycnium rectum</i> . . .	54·5	1·5	44·0
<i>Lotus Requiinii</i> . . .	44·0	3·0	53·0
<i>Medicago sativa</i> . . .	41·2	0·5	58·2
<i>Trifolium montanum</i> . . .	40·0	19·0	41·0
<i>Trifolium rubens</i> . . .	28·0	13·0	59·0
<i>Trifolium medium</i> . . .	27·2	23·2	49·5
<i>Lotus ornithopodioides</i> . . .	20·5	4·0	75·5
<i>Lotus uliginosus</i> . . .	18·0	1·0	81·0
<i>Trifolium ochroleucum</i> . . .	16·0	2·0	82·0
<i>Melilotus albus</i> . . .	14·0	1·0	85·0
<i>Ononis arvensis</i> . . .	9·0	12·0	79·0
<i>Lotus corniculatus</i> . . .	8·2	8·5	83·2
<i>Ononis spinosa</i> . . .	7·0	3·0	90·0
<i>Medicago falcata</i> . . .	5·2	0·5	94·2

were hardened, however, by the process of dehydration so that they cut no more easily than the dry ripened seeds. The specimens used for illustration were the best typical parts and were drawn with the aid of a camera lucida from sections  $4\mu$  in thickness. To give an indication of the nature of the substances present in each part of the seed coat, microchemical tests were made by using standard reagents on fresh material to detect the presence of cellulose, lignin, cutin, suberin, callose, and pectin.

#### *Germination tests*

To obtain germination percentages representative of the tribes Trifoliae and Loteae, several tests were made on a larger group of species.

Having established from preliminary experiments the fact that the exclusion of light had no effect on the percentage or rate of germination of six species, the following experiment was carried out under ordinary daylight conditions in a cool greenhouse at a temperature of about 60° F.

Four separate germination tests, of 35 days' duration, were made at various times over a period of 18 months. Twenty-one species of representative plants

from the tribes Trifoliae and Loteae were chosen. The results for these tests are summarized in Table I; the species are arranged in order of germination merit. Although the percentage of mouldy seeds is high in some species, in no case does it prevent seeing whether a large or small percentage of the seeds remains impermeable. Seven species have a percentage of germination higher than 60, and may be termed permeable seeds of good germinating power. Eight species have a percentage of non-germination higher than 75, and may be termed impermeable seeds of poor germinating power. The percentage of impermeability ranges from zero in *Anthyllis tetraphylla* to 94 in *Medicago falcata*, and the percentage of germination ranges from 98 in *Trifolium hybridum* to 5 in *Medicago falcata*.

### Seed coat prevents absorption

An experiment was conducted to prove that the seed coat prevented the absorption of water in the impermeable species of the plants under observation. A small opening was filed on each seed of samples from the eight most impermeable species. These seeds were placed under germinating conditions along with a control sample of whole, unfiled seeds. After 48 hours every filed seed had either germinated or swollen considerably, while only a very small percentage of unfiled seeds had shown any signs of permeability. All the seeds which were filed had a percentage impermeability of zero after 5 days under conditions suitable for germination. The unfiled seeds had an average percentage impermeability of 78.5 (Table II).

TABLE II

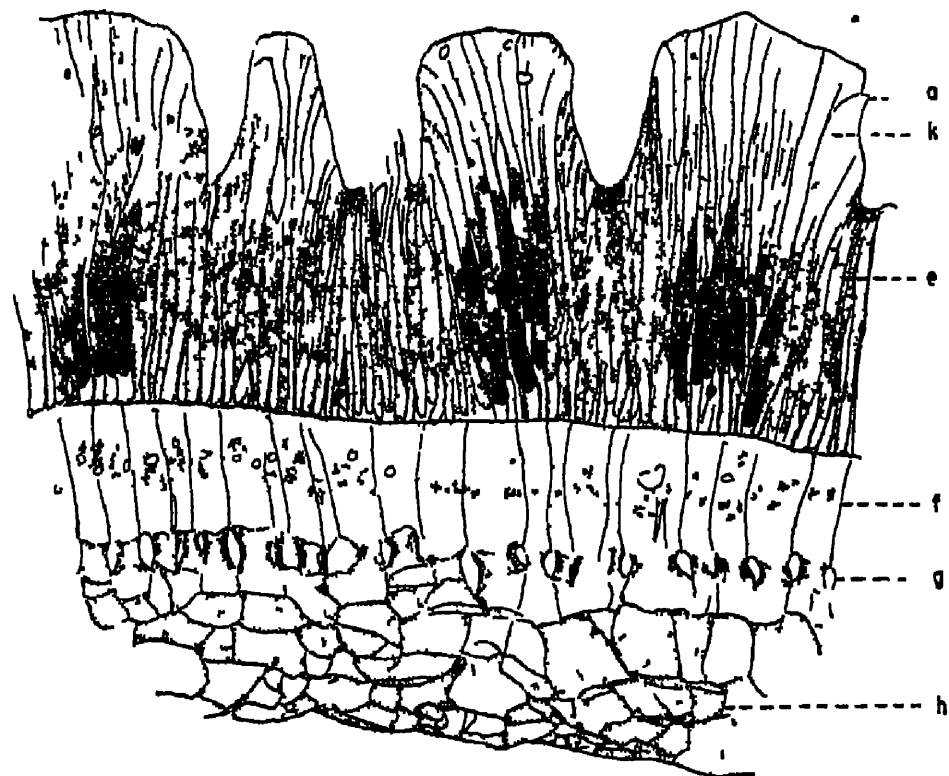
*Filing the Seed Coat of Impermeable Seeds causes them to become Permeable and to germinate (after Five Days)*

	Number in percentage							
	Not filed				Filed			
Impermeable species.	Germina- ted.	Swollen.	Mouldy.	Imper- meable.	Germina- ted.	Swollen.	Mouldy.	Imper- meable.
<i>Lotus ornithopodioides</i> .	36	2	4	58	98	0	2	0
<i>Lotus uliginosus</i> .	8	8	0	84	70	30	0	0
<i>Lotus corniculatus</i> .	6	8	0	86	98	0	2	0
<i>Ononis spinosa</i> .	4	2	2	92	98	0	2	0
<i>Ononis arvensis</i> .	12	4	2	82	92	0	8	0
<i>Trifolium ochroleucum</i> .	52	0	0	48	100	0	0	0
<i>Melilotus albus</i> .	20	0	0	80	100	0	0	0
<i>Medicago falcata</i> .	2	0	0	98	100	0	0	0

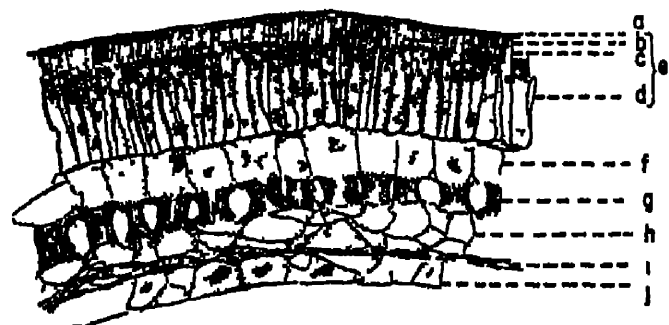
### DESCRIPTION OF STRUCTURE OF TESTAS IN RELATION TO GERMINATION

#### 1. *Anthyllis tetraphylla* (Fig. 1)

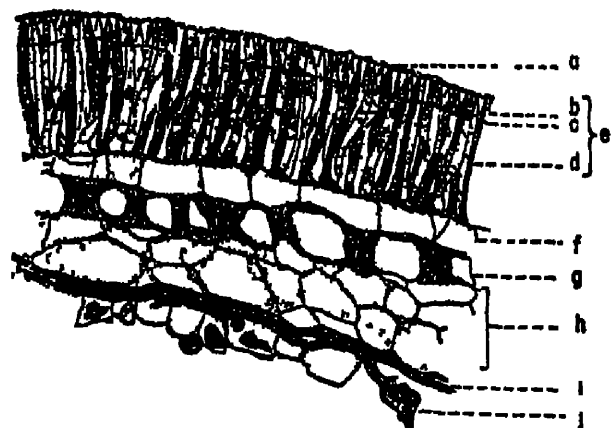
This is the largest seed used in this study. Its general structure is similar to others, but there are small deviations. The cuticle, covering an extremely uneven seed surface, is quite thin and inconspicuous. The uneven raised



1. *Anthyllis tetraphylla*



2 *Dorycnium rectum*



3. *Lotus Requierii*

FIG. 1. Drawings of transverse sections of testas *a*, cuticle; *b*, sub-cuticular layer; *c*, light line; *d*, lower portion Malpighian cell; *e*, Malpighian cell; *f*, intermediary layer, *g*, osteoscleroid cell; *h*, nutrient layer, *i*, collapsed nutrient layer; *j*, aleurone layer, *k*, raised portion. (X 240.)

portions reach half-way into the Malpighian layer. As the long, narrow, and somewhat entangled cellulose Malpighian cells stretch out into these protrusions, their outline becomes indistinct. The inner boundary of the sub-cuticular layer is not determinable because of the absence of a light line. Towards the inner end of the Malpighian cells there are thickened areas and deposits of cutin. The osteosclereid cells are long and filled with cellulose. They are constricted about two-thirds of the way in, thickened at this point with cutin, and separated by small, prismatic or oval intercellular spaces. There is a deep layer of large, clear nutrient cells which are composed almost entirely of cellulose. The aleurone layer rarely adheres to the nutrient layer in cutting.

This species of *Anthyllis* has seeds of particularly good germinating power, the percentage of germination being 88. There is no heavy deposit of cutin or suberin to prevent the absorption of water. The cellulose Malpighian cells and the very small deposit of cutin in the osteosclereid thickening may be the reason that so many of these seeds are permeable to water.

## 2. *Dorycnium rectum* (Fig. 1)

There is a fairly thin cuticle on the seed coat of *Dorycnium rectum*. Above the light line, which seems to consist mostly of cellulose with a very small quantity of cutin, there are no distinct cell limits, but rather heavy deposits of cellulose with lines running through them. This region contains a very little cutin. The lines continue through the comparatively wide light line into the inner parts of the Malpighian cells. Directly under the light line there is a little cutin, but most of the cell-wall below it is cellulose and callose in nature. The intermediary layer, which is composed of cutin, is probably just the outer parts of the cuticularized osteosclereid cells. The osteosclereid cells reach down into the nutrient layer in places, are opaque, and do not contain strands as in some species. Their intercellular spaces are large and prism-shaped. The nutrient layer of more or less clear cells often partly collapses next to the large, regular, cellulose aleurone cells, and leaves a very narrow nutrient region.

On the testa of *Dorycnium rectum* there is no very great cuticular thickening preventing the absorption of water except in the constricted portion of the osteosclereid cells. The percentage of germination is 54, which shows that water must enter a large number of the seeds.

## 3. *Lotus Requiensi* (Fig. 1)

A more detailed description of this seed coat may be found under 'Description of a Leguminous Testa'.

The cuticle is composed of cutin; the sub-cuticular layer of suberin and callose. In the light line there is an even deposit of cellulose with a little callose. The lower Malpighian cells consist mostly of cellulose with small grains of cutin. Although the osteosclereid cells contain mostly cellulose, they also give a slight reaction for suberin. The walls of the large clear nutrient

cells are cuticularized, and there is a small quantity of cutin in the aleurone layer.

Although the deposits of suberin in the sub-cuticular layer and osteosclereid cells might prevent the absorption of water in some seeds, there is no heavy cuticularized region. The percentage of germination is 44.

#### 4. *Lotus uliginosus* (Fig. 2)

The cuticle on the testa of *Lotus uliginosus* is of medium thickness. There are no distinct caps. The sub-cuticular layer contains cellulose, pectin, cutin, and suberin in comparatively small quantities. The light line is indistinct and gives a very slight positive test for cellulose. Beneath the light line, the Malpighian cells are regular in form but so well filled with pigment that staining is unreliable. The intermediary layer may or may not be present. The osteosclereid cells are broad, spool-shaped, and extend from the base of the Malpighian cells down into the nutrient cells. The osteosclereid cells, containing cutin and pectin where they are thickened through the centre of each cell, are separated by small, prismatic intercellular spaces. The large, clear nutrient cells give slight cutin and pectin reaction, and are often present in a collapsed state. The aleurone layer, although broken away in some sections, is quite regular and contains cutin when it is present.

This seed has a germination percentage of only 18, yet the amount of cuticular thickening does not appear to be very great. Although the structure of the sub-cuticular layer is similar to that of many testas which have well-capped Malpighian cells, no distinct cuticular layer of caps is present to prevent the absorption of water.

#### 5. *Lotus corniculatus* (Fig. 2)

*Lotus corniculatus* has a fairly thick cuticle which is composed of pectin as well as cutin. The sub-cuticular layer is made up of a row of pointed, suberized Malpighian tips which contain a trace of cellulose. The light line is a clear, narrow band of cutin, but the longitudinal walls of the Malpighian cells show through it. The Malpighian cells are a dark yellow colour below the light line. They are long, small in diameter, and somewhat entangled one with another. There is a distinct intermediary layer of large, clear cells with cuticularized cell-walls. The osteosclereid cells are wide, clear, and thickened with cellulose and pectin. They have small intercellular spaces. The nutrient layer is made up of large, clear cells with odd particles of cutin and cellulose in them. They are smaller and more collapsed near the aleurone layer. The aleurone layer consists of cellulose and is broken away in part.

The deposits of cutin in the cuticle and light line would not seem sufficient to cause the seed of *Lotus corniculatus* to be impermeable and consequently to have the low germination percentage of 8. Although the cellulose tips do not form an impermeable layer, the presence of suberin in these tips suggests a line of impermeability which may prevent the absorption of water.

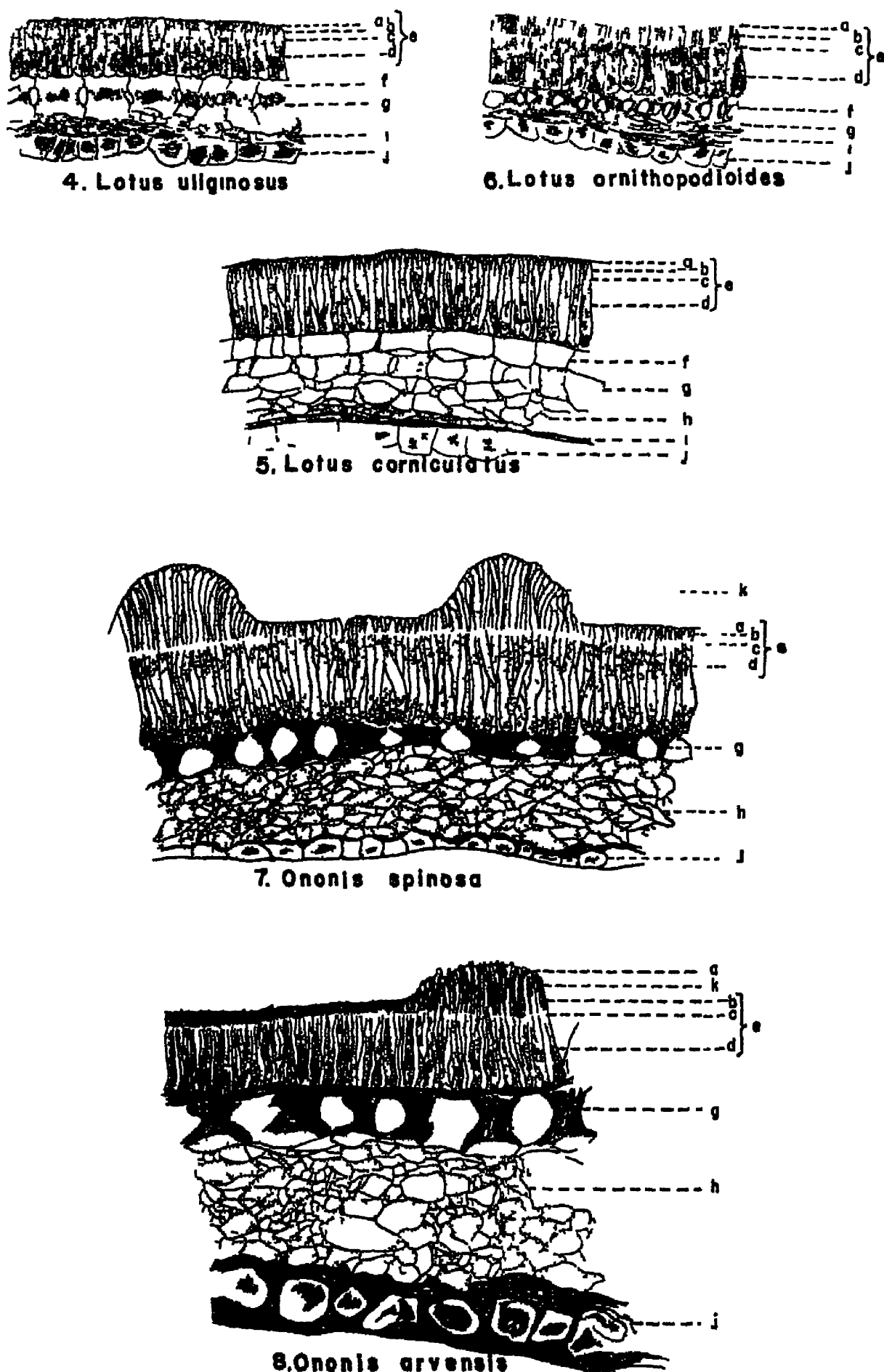


FIG. 2. Drawings of transverse sections of testas. *a*, cuticle; *b*, sub-cuticular layer, *c*, light line; *d*, lower portion of Malpighian cell, *e*, Malpighian cell; *f*, intermediary layer; *g*, osteosclereid cell; *h*, nutrient layer, *i*, collapsed nutrient cells; *j*, aleurone layer; *k*, raised portion (tubercles). ( $\times 240$ .)

6. *Lotus ornithopodioides* (Fig. 2)

The cuticle is indistinct and fairly thick on *Lotus ornithopodioides*. The sub-cuticular layer forms a band in which some callose is present. There are also many cuticularized lines or canals in the subcuticular layer, and these cross the clear, narrow light line. The boundaries of the individual Malpighian cells cannot be determined in the band. The cuticularized light line fades at its edges but is distinctly visible. The walls of the Malpighian cells are clearly visible inside the light line, and they contain cellulose and a trace of suberin. It has not been possible to see clearly whether the osteosclereid cells extend from the inner end of the Malpighian layer to the nutrient layer, whether there is an intermediary layer of entire cells directly above the osteosclereid cells, or whether the osteosclereid cells separate from the Malpighian cells when the sections are being cut. Inconspicuous strands of cellulose and a little callose run longitudinally through each osteosclereid cell. In most sections the nutrient cells are not clearly visible but simply seem to be a badly collapsed mass of cellulose. The aleurone layer is quite regular and has a high cellulose content.

*Lotus ornithopodioides* is a species which germinates poorly, its germination percentage being 20. The thick deposits of cutin which have been found in the light line, sub-cuticular layer, and cuticle might prevent the absorption of water by the seed.

7. *Ononis spinosa* (Fig. 2)

The cuticle on the seed of *Ononis spinosa* is thin and slightly uneven. The Malpighian cells are extremely long and pass through the sub-cuticular layer into the raised tubercles which are on the surface of the seed. Above the light line, the Malpighian cells are mostly composed of cellulose, although in the tips of some cells, especially in the raised portion, there is a little cutin present. The light line is not conspicuous, but the longitudinal walls of the Malpighian cells are clearly visible through it. The Malpighian cells contain cellulose and a trace of cutin below the light line, but the light line consists entirely of cellulose. There are large intercellular spaces between the cellulose osteosclereid cells. The nutrient layer is a confused mass of protoplasm made up of cells of various sizes, which contain a little cutin. The aleurone layer is one row of cuticularized cells which contain a trace of callose.

There is no great thickening in the outer part of this testa to prevent the absorption of water. The Malpighian cells are composed of cellulose for the most part, and the seed would seem to be easily penetrated. Although there is no apparent thickening to prevent the absorption of water, the percentage of germination is only 7.

8. *Ononis arvensis* (Fig. 2)

The cuticle on the seed of *Ononis arvensis* is usually found intact after a section has been cut. There are no caps on the Malpighian cells, but there is a thickening above the light line in the sub-cuticular layer, in the form of a

deposit of cellulose and a little cutin and suberin. This is found in the tips of the cells in the raised tubercles. The walls of the Malpighian cells are visible through the wide but indistinct light line. The Malpighian cells are long and, like the light line, contain cellulose. There is a dark band of demarcation at the base of the Malpighian cells. The osteosclereid cells, which are composed of indistinct strands of cellulose, contain a little callose in some places. Although the strands and cell-walls are present in most sections, they are not always visible. There are large intercellular spaces between the osteosclereid cells. The nutrient layer is quite deep and made up of various-sized clear cells which contain a slight deposit of cutin and callose. The aleurone layer, being cuticularized, is very thick and conspicuous.

There is no great thickening of cutin in this testa to prevent the absorption of water. Between the small cuticularized portions, whether in the tubercles or other places in the sub-cuticular layer, there are large deposits of cellulose which are quite free to absorb water. The cellulose Malpighian cells without caps and without a line of cutin indicate a good percentage of germination instead of the small percentage of 9 which has been obtained.

9. *Trigonella caerulea* (Fig. 3)

*Trigonella caerulea* produces a seed which has a cuticle of uneven thickness. There is a deep, cellulose sub-cuticular layer with a trace of callose in it. Embedded in this matrix is a row of cuticularized caps on the tips of the Malpighian cells. The light line is quite indistinct, so much so that it cannot be accurately stained. The longitudinal walls of the Malpighian cells cross it. The Malpighian cells are broad and well filled with yellow pigment below the lightline. The osteosclereid cells, which consist of cellulose, have the appearance of being stretched out, making them long and sheaf-like, with large spaces between the cells. The osteosclereid cells contain a trace of callose. The wide nutrient layer is comprised of large, rather clear cells which have scattered particles of cutin, cellulose, and callose in them. There is very little collapsed nutrient tissue, but a regular cuticularized aleurone layer which contains some callose.

*Trigonella caerulea* has a germination percentage of 81. Although there is a perfect row of cuticularized Malpighian caps which appear to be joined at their base, there is a possibility that the cutin is not as effective in preventing the absorption of water as in a species which has a higher percentage of germination. The structure of this testa is, however, very similar to some of the species of poorer germination.

10. *Medicago lupulina* (Fig. 3)

There is a thick cuticle on the seed coat of *Medicago lupulina*. It breaks away very easily in cutting. There are small, narrow-tipped, cuticularized, and suberized Malpighian caps in the sub-cuticular layer. The caps also contain a trace of pectin. The matrix in which the caps are embedded is mostly cutin, but does contain a trace of cellulose. Most of the Malpighian

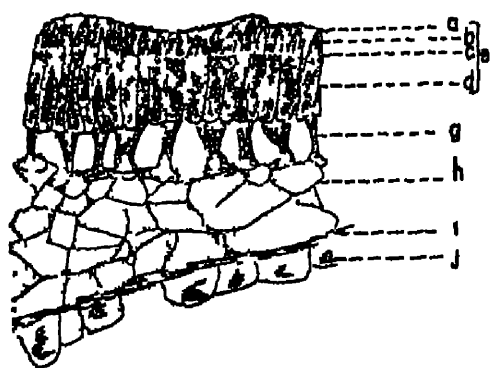
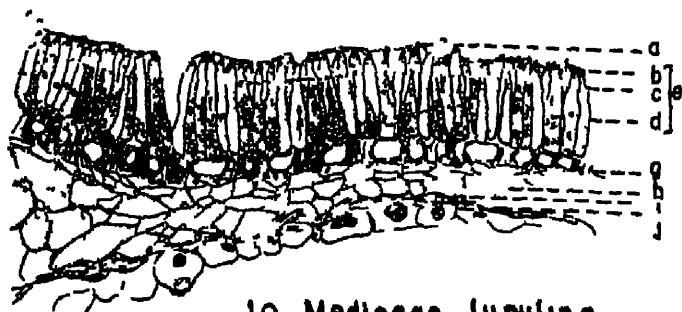
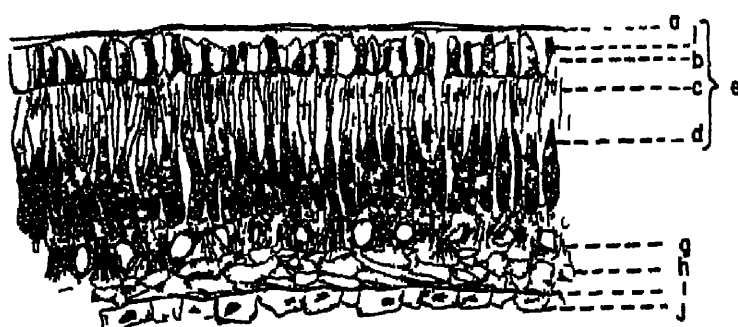
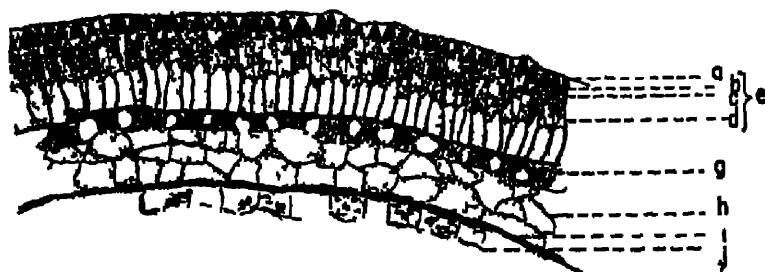
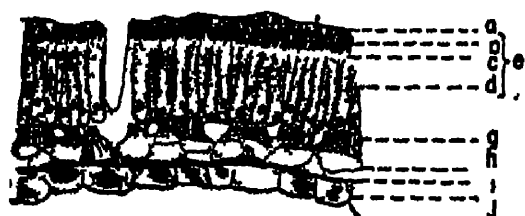
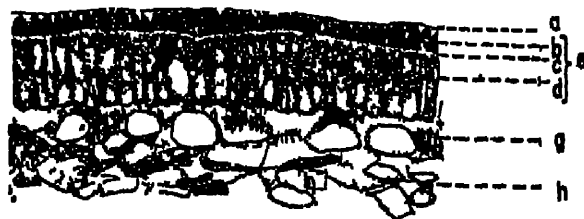
9. *Trigonella caerulea*10. *Medicago lupulina*11. *Medicago cuneata*12. *Medicago sativa*13. *Medicago falcata*14. *Melilotus albus*

FIG. 3. Drawings of transverse sections of testas. *a*, cuticle; *b*, sub-cuticular layer; *c*, light line; *d*, lower portion of Malpighian cell; *e*, Malpighian cell; *f*, intermediary layer; *g*, osteoscleroid cell; *h*, nutrient layer; *i*, collapsed nutrient cells; *j*, aleurone layer; *l*, tooth-like structures. ( $\times 240$ .)

cells are well filled with cellulose and suberin below the wide, indistinct, suberized light line, which is crossed by Malpighian cell-walls. The osteosclereid cell-walls contain cutin and faint strands of cellulose and callose. Their intercellular spaces are usually quite large. There is a medium-sized nutrient layer of mostly clear cells of cellulose and callose, at times collapsed and showing an inner layer of cutin. The aleurone layer is an uneven row of cuticularized and suberized box-like cells.

Although there are small, cuticularized caps on the tips of the Malpighian cells, they do not extend far enough into the tips to be united at their base and form a line of impermeable material. The germination percentage of *Medicago lupulina* is 62, so that the water must enter a number of the seeds fairly easily. The cutin in the matrix may not be as efficient in preventing the absorption of water as in some species which have a low percentage of germination.

11. *Medicago cuneata* (Fig. 3)

The cuticle on the seed of *Medicago cuneata* is clear and thin and contains, as well as cutin, a trace of callose. Under the cuticle there is a layer of tooth-like structures which are not found in any of the other seeds which have been examined. The disappearance of the Malpighian cell-walls as they pass through the light line makes it impossible to tell whether one of these structures forms a tip for each Malpighian cell. These tooth-like structures contain a small quantity of suberin, cutin, and callose, but they are very difficult to stain. The long Malpighian cells contain sac-like protoplasm which stretches up to the light line in some places. The osteosclereid cells are broad and separated by clear intercellular spaces. These cells are filled with cellulose strands which extend down into the nutrient layer in some places. The nutrient layer is made up of small cells which contain a little cutin and callose. The aleurone layer is a regular row of cuticularized cells.

*Medicago cuneata* has a germination percentage of 69. There is no dense thickening to prevent the absorption of water. The small amount of cutin in the cuticle, sub-cuticular layer, and light line is evidently not effective in preventing the entrance of water into the seed.

12. *Medicago sativa* (Fig. 3)

The cuticle on the seed of *Medicago sativa* is not clearly visible. There are cuticularized Malpighian caps in the sub-cuticular layer which contain a trace of pectin. The composition of the light line has not been ascertained. It does not change its colour after being tested for either cutin or cellulose, but remains transparent with lines indistinctly showing through it. The inner part of the Malpighian cell is broad in diameter, quite yellow, and contains some cellulose. The organic nature of the strands of the wide, flat osteosclereid cells has not been ascertained because they continue to remain transparent after treatment with all the reagents which have been employed. The nutrient cells are large and clear. In them there is a trace of cutin and a patchy deposit

of cellulose particles. Between the nutrient layer and the cuticularized aleurone layer is a row of collapsed nutrient cells. The aleurone layer is partly broken away in many sections.

*Medicago sativa* has a germination percentage of 41. The line of cuticularized caps in the sub-cuticular layer may prohibit the water from penetrating the inner walls of the testa and the rest of the seed. Although the structure of the cuticularized caps seems very similar to that found in *Melilotus albus*, *Melilotus officinalis*, and *Trigonella caerulea*, it is possible that the thickening of cutin may not be as efficient in preventing the absorption of water as it is in some of the poorer germinating species.

### 13. *Medicago falcata* (Fig. 3)

The seed of *Medicago falcata* has a thin cuticle which breaks away very easily when the sections are being cut. On the tips of the long Malpighian cells there are broad cuticularized and suberized caps which, being united at their bases and rounded at their tips, form an uninterrupted line. The light line is clear and thin, with no lines crossing it. The light line and all the structures outside of it contain cutin. Although the lower portion of the Malpighian cells, which are filled with cellulose, are often separated one from another in cutting, they are held together at the outside tip by the Malpighian caps. The osteosclereid cells, which contain a little cutin, have cellulose strands running through them. There are large intercellular spaces between each osteosclereid cell. The nutrient layer, showing a trace of pectin and some cellulose, is two or three almost clear cells in depth. Towards the cuticularized aleurone layer the nutrient cells have collapsed.

The cuticularized portions of the cuticle, caps, and light line evidently prevent the absorption of the water by the seed, for it is found that the seed of *Medicago falcata* has a germination percentage of only 5. There appears to be no cellulose present between the light line and the outside of the seed, so the water has to pass the cuticularized portions before it may reach the cellulose and be more readily absorbed.

### 14. *Melilotus albus* (Fig. 3)

The cuticle on the seed of *Melilotus albus* is quite thin but usually intact after the seed has been sectioned. There are suberized and cuticularized, more or less pointed caps on the Malpighian cells. These Malpighian caps are joined together at their bases. Between the caps the sub-cuticular matrix is cellulose. The light line is a clear band of cellulose with no lines crossing it. The wider portions of the Malpighian cells, which are below the light line, are difficult to stain because of the presence of deep yellow pigment. The osteosclereid cells are difficult to see. They may easily be confused in places with the nutrient cells. Where the osteosclereid cells are distinctly visible they are small and have bands of finger-like marks in them. The osteosclereid cells, containing cutin and a trace of callose, are separated by large, uneven intercellular spaces. The nutrient layer is made up of large cells which contain

cellulose, cutin, and a trace of callose. They collapse towards the aleurone layer, which is broken away in most sections.

The line of suberized and cuticularized Malpighian caps may prevent the water from entering most of the seeds of *Melilotus albus*, for its percentage of germination is only 11.

15. *Melilotus officinalis* (Fig. 4)

The seed of *Melilotus officinalis* has a fairly thick cuticle which is broken away in cutting the sections. The bluntly rounded tips of the long, thick-walled Malpighian cells are in the form of cuticularized and suberized caps. These contain traces of pectin, and are united at their bases to form an unbroken line around the seed. There is cutin in the cuticle, in the sub-cuticular layer between the caps, and in the clear light line. The Malpighian cell-walls cross the light line and become thickened in the lower portions of the cellulose Malpighian cells. Strands are visible on most of the cellulose osteosclereid cells. In some sections the osteosclereid cells are much more conspicuous than in others. The nutrient cells are large and clear, and have a trace of callose and pectin. The aleurone layer is a single row of thick-walled, cuticularized cells directly under the collapsed nutrient cells.

The deposits of suberin and cutin apparently form a completely impermeable line which seems sufficient to prevent any absorption of water, nevertheless the percentage of germination is 62. This testa is very similar in structure to that of *Melilotus albus*, which germinates very poorly.

16. *Trifolium montanum* (Fig. 4)

The cuticle on the seed of *Trifolium montanum* is thin and has a very uneven surface. The semi-caps of cutin and pectin occupy a large portion of the cuticularized sub-cuticular layer. The light line is wide and indistinct, with very thick Malpighian walls below it and a few showing through it. Malpighian cells are broad and somewhat cuticularized. On the osteosclereid cells the cell-walls are very conspicuous, but the cells are flat and have no strands running through them. They contain cutin and are separated by large inter-cellular spaces. The nutrient cells also contain small quantities of cutin, but they are large, clear cells, and many have collapsed just outside the aleurone layer. The aleurone layer is a row of long, narrow, cuticularized cells.

All the cutin except that in the cuticle, sub-cuticular layer, and light line is in small quantities. It is probable that water is not prevented from entering the seed to nearly as great an extent as if the cutin were deposited in an impermeable, unbroken line. This may account for the germination percentage of 40 which *Trifolium montanum* possesses.

17. *Trifolium pratense* (Fig. 4)

On the seeds of *Trifolium pratense* the cuticle is thin and breaks away easily when the sections are being cut. There are no Malpighian caps present, but in the sub-cuticular layer cuticularized and suberized joined strands or canals

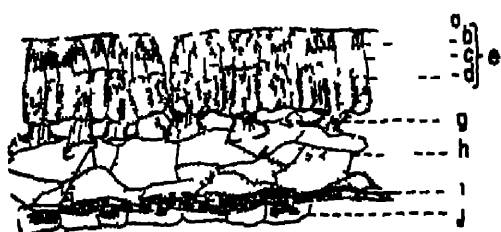
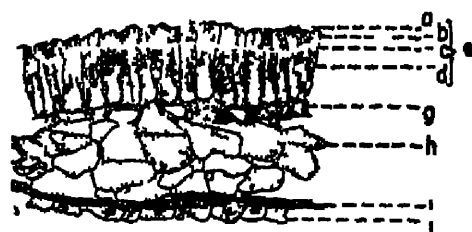
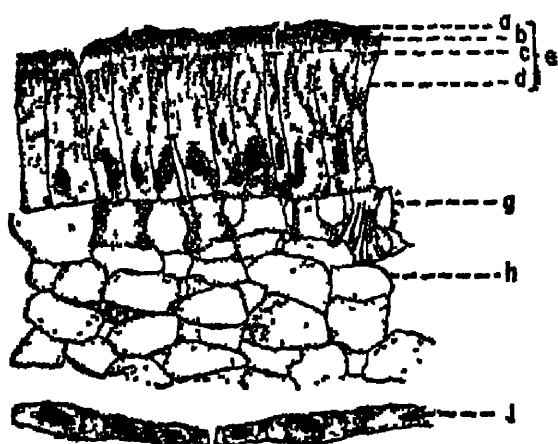
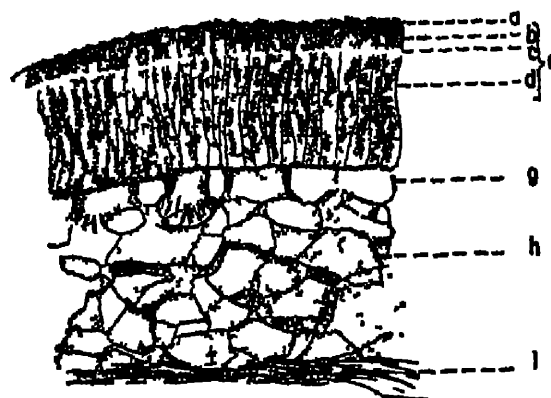
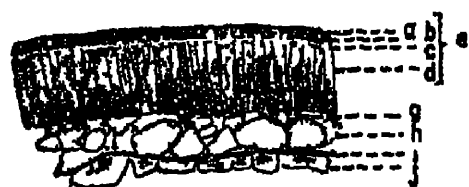
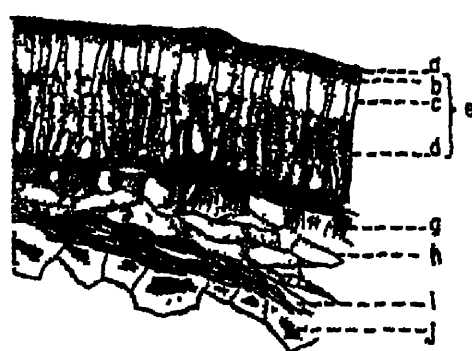
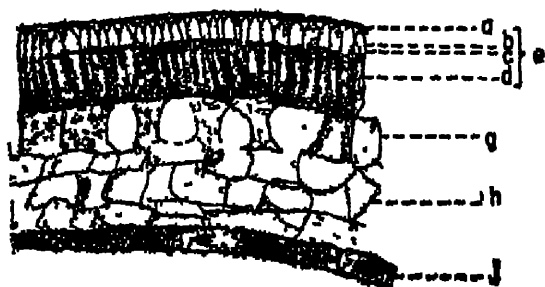
15. *Melilotus officinalis*16. *Trifolium montanum*17. *Trifolium pratense*18. *Trifolium ochroleucum*19. *Trifolium rubens*20. *Trifolium medium*21. *Trifolium hybridum*

FIG. 4 Drawings of transverse sections of testas. *a*, cuticle, *b*, sub-cuticular layer; *c*, light line; *d*, lower portion of Malpighian cell; *e*, Malpighian cell; *g*, osteoscleroid cell; *h*, nutrient layer; *i*, collapsed nutrient cells; *j*, aleurone layer. ( $\times 240$ .)

form a layer directly under the cuticle. These strands also contain a trace of pectin. The light line is not conspicuous; cross lines show through it. Distinct canals run from the light line into the lower part of the broad Malpighian cells. The lower portion of the Malpighian cells contains cellulose, but the light line does not stain. In many sections the osteosclereid strands are not conspicuous, but the spool-shaped outline of the cells is indicated. The nutrient layer consists of a mass of clear cells of different sizes and shapes containing traces of pectin and cutin. The aleurone layer, which is also cuticularized, is not attached to the nutrient layer, but clings to the cotyledons in most seeds.

Although there are deposits of cutin in the strands of the sub-cuticular layer, they do not form a complete line which might prevent the absorption of water by the seed. The light line and lower portion of the Malpighian cell contain no cutin. This species has a germination percentage of 60.

18. *Trifolium ochroleucum* (Fig. 4)

On the seeds of *Trifolium ochroleucum* there is a thin cuticle. The sub-cuticular layer contains cutin, suberin, and a trace of callose, and in places the tips of the Malpighian cells cross and make occasional pieces thick. There are no Malpighian caps on this testa, but suberized tips. The light line is wider in some seeds than in others, but in every one it is cutinized and the longitudinal walls of the cells show through it. The Malpighian cells are long and form a somewhat entangled mass of cellulose with some canals in them. The cellulose osteosclereid cells are large and opaque with no strands in them. The osteosclereid cells are separated by large intercellular spaces. The nutrient cells are clear, large, and composed of cellulose and a little cutin. They have collapsed towards the inside of the testa, and there is no aleurone layer clinging to the nutrient layer in most of the sections.

On *Trifolium ochroleucum* there is no very great thickening of cutin, but its presence in the cuticle, sub-cuticular layer, and light line, along with the suberized Malpighian tips, suggests the cause of the low germination percentage of 16.

19. *Trifolium rubens* (Fig. 4)

There is a fairly thick cuticle containing a trace of pectin on the seed of *Trifolium rubens*. At times it is broken away. The blunt tips of the Malpighian cells do not form caps, but are cuticularized and contain a very little callose. The light line is narrow, transparent, and gives a positive test for callose. The Malpighian cell-walls show through it very faintly. The Malpighian cells, which are regular, are filled with cellulose and a little callose. They are tangled towards their bases. Although the osteosclereid cells are mostly cellulose, they contain a slight amount of callose. In most sections these cells are not very prominent, but in many they may be distinguished from the large nutrient cells which are composed of the same materials. Towards the aleurone layer the nutrient cells are crushed and form a thin layer. The aleurone layer is an uneven row of cuticularized cells with a little callose in them.

The deposit of cutin towards the outside of the testa does not seem to be very heavy, and yet the germination percentage for *Trifolium rubens* is only 28, an indication that the water does not enter many of the seeds.

20. *Trifolium medium* (Fig. 4)

The Malpighian cells reach out to the fairly thick cuticle, maintaining about the same diameter at their apex as at their base. They have a cuticularized thickening directly under the cuticle, but it is an even deposit rather than a cap formation. There is an extremely wide, clear light line with the longitudinal walls of the Malpighian cells showing through it. The osteosclereid cells are wide, flat, and cuticularized, having a slight indication of strands running through them. At the outer edge where they join the inner extremity of the Malpighian cells they form a thick band. The nutrient layer, being nearly all collapsed, is only a few clear cells in width. The aleurone layer as well as the nutrient layer is cuticularized, but the aleurone cells are larger, have thicker walls, and are in a more regular formation.

*Trifolium medium* has a germination percentage of 27. The cuticular deposits which are present throughout the whole testa may prevent the absorption of water in a great number of the seeds. Although the amount of deposit varies from one structure to another, it is no doubt thick enough in the sub-cuticular layer to prevent the absorption of water by many of the seeds.

21. *Trifolium hybridum* (Fig. 4)

The clear tips on the Malpighian cells, containing a small quantity of suberin and pectin, are covered with a thin distinct cuticle of the same materials. The matrix between these tips in the sub-cuticular layer also contains a small quantity of cutin. The light line is indistinct, but apparently is quite wide. The walls of the Malpighian cells are distinctly visible across the light line. Towards the inner end of the Malpighian cells there is a wall thickening of cutin. Cellulose, cutin, suberin, and callose reactions are obtained all through the osteosclereid and nutrient cells. The aleurone layer shows regular cuticularization.

The suberized and cuticularized thickening in the Malpighian cells seems sufficient to prevent the water from being absorbed by the seed, but *Trifolium hybridum* has the excellent percentage germination of 98. In this species, as in some others, the thickening may be less effective in preventing the absorption of water than in a species with a lower percentage of germination.

### CONCLUSIONS

An attempt which has been made to link up the wide range of germination percentages with the structure and chemical composition of the testa shows that no structure which prevents the absorption of water can be found in every species with a low percentage of germination. The amount of suberized and cutinized sub-cuticular and Malpighian thickening found in each species of

the whole group of seeds seems to be sufficient to justify the high general rate of impermeability throughout.

This group of seeds, which has been subjected to the same treatment in every way, may be conveniently separated into the seven best germinating species (above 60 per cent.), the eight poorest germinating species (below 21 per cent.), and the six which are not definitely poor or good germinating species (between 27 and 55 per cent.). Of the eight poorest germinating species there are three, *Ononis spinosa*, *Ononis arvensis*, and *Lotus uliginosus*, whose structure indicates that they absorb water freely. Of the seven best germinating species there are three, *Melilotus officinalis*, *Trigonella caerulea*, and *Trifolium hybridum*, whose structure indicates that they do not absorb water freely. If there is any relation whatever between the structure and chemical nature of the seed coats and the germination of the seeds, the same suberized or cuticularized deposits must be more effective in preventing the absorption of water in some species than in others.

There is a marked uniformity in the structure of the testa of each species within one genus but, although both species of *Ononis* and three species of *Lotus* have a low percentage of germination, there is no other similarity between the percentage germination of different species of the same genus.

White (1908) has given illustrations to show how the thickness of the cuticle affects the amount of water absorption in a number of species of seeds. In the twenty-one species examined in this work the variation in the thickness of the cuticle is so small that it is very difficult to record and could in no way be linked with the degree of impermeability of a seed.

Thickened, suberized, or cuticularized Malpighian caps, as were described by Hambly (1932), have been found on the seed of *Melilotus albus* which has a germination percentage of 14, but caps of a very similar nature are also found on *Melilotus officinalis*, a species which has a germination of 62 per cent. Malpighian caps are present therefore on seeds of both low and high germinating percentages. *Trigonella caerulea* and *Trifolium hybridum* have cuticularized tips similar to those of *Melilotus*, and yet a high percentage of germination. At least two of the seven most permeable species and two of the most impermeable species have caps.

The cells of the aleurone layer have been found to be thin-walled in all species with shrunken cell contents, rather than thick-walled as was reported by Hambly (1932) in *Melilotus albus* and Pammel (1899) in other species.

Coe and Martin (1920) have suggested that it was not the sub-cuticular layer or the Malpighian caps but the absence of canals in the light line, and more intense thickening below the light line, which prevented the absorption of water in *Melilotus albus* and *Melilotus officinalis*. No particular type of light line may be selected from the various species which have been examined at this time as being characteristic of all the species with a low percentage of germination, but there is usually a more clear narrow line in most of the less permeable species. No general conclusion of its relation to permeability can be made, but a narrow line without cross lines is least common. The light

line appears to contain cutin in some species and cellulose in others, but in many it remains clear when stained with the microchemical reagents employed. It is possible that the colour of the neighbouring material might be reflected in the light line and make accurate results difficult to obtain. The appearance of the light line is greatly affected by different types of light, and to make a definite statement of its nature is dangerous without a more intense study under various types of illumination.

Most of the thickening in the lower portion of the Malpighian cells is cellulose, but cutin or suberin is found to be present in both the permeable and impermeable species.

The remaining structures from the osteosclereid cells into the aleurone layer are in most instances constructed of more permeable materials. No heavy deposits of cutin are found except in the aleurone layer. Probably the most common substance and certainly the one most heavily deposited in these regions is cellulose. The presence of cutin or suberin is not infrequent, and when found is mostly in odd grains or thin, even deposits. The most impermeable area of the testa is no doubt outside of the inner ends of the Malpighian cells.

This analysis explains the disagreement among former investigators working with different species at various times. As the percentage of germination increases from one species to another, there is no definite coherent trend towards uniformity of the chemical or structural nature of any one particular region within the various species which have been examined. No structural feature causing impermeability in the testa can be shown to be present in all the impermeable and none of the permeable seeds.

#### SUMMARY

A résumé of the previous work by other investigators deals with the structural causes of, and the methods used to break down, impermeability.

The structure of the testas of representative species of the sub-families Loteae and Trifoliae has been analysed to see if it has any relation to the percentages of germination obtained in a series of tests.

Filing a small crack on the most impermeable species proved that the seed coat was preventing the absorption of the water.

The average germination of each species in several tests varies from 5 up to 98 per cent. There are seven permeable species which germinate over 60 per cent. and eight impermeable species which germinate below 21 per cent.

The detailed description of the testas indicates that there is a great variation in the structure and chemical nature of the seed coats in question.

The amount of suberized and cuticularized sub-cuticular and Malpighian thickening found in each species of the whole group seems sufficient to justify a high general rate of impermeability throughout.

As the percentage of germination increases from one species to another there is no definite coherent trend towards uniformity of the chemical or structural nature of any one particular region within the species examined.

No structural feature causing impermeability in the testa can be shown to be present in all the impermeable and none of the permeable seeds.

#### ACKNOWLEDGEMENTS

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The Continuity of Intercellular Spaces in the Leaf of  
Pelargonium zonale, and its Bearing on Recent  
Stomatal Investigations

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With two Figures in the Text

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INTRODUCTION

THE leaf of *Pelargonium zonale* var. *Paul Crampel*, which has been extensively used for stomatal investigation, is entire or slightly lobed, with prominent palmate venation. Each sector enclosed by two main veins is homobaric, i.e. the intercellular spaces are continuous throughout the sector; but it has been generally assumed that the intercellular space systems of *adjacent* sectors are isolated from each other by the main veins. Heath (1941), for example, states that 'it is probable that the main veins provide an almost complete barrier to gas movement either by viscous or diffusive flow'.

The matter is of little intrinsic interest, but it has a direct bearing on two lines of current stomatal investigation:

1. In any work in which more than one porometer-cup is attached to the same leaf, such as that of the present author on heat-shock transmission (Williams, 1948), it is necessary to postulate not only that the two groups of stomata behave independently, but also that the air-streams passing through them are independent; if this is not the case a change in aperture of one set of stomata will result in a change of porometer reading for the other set, even though there may have been no corresponding stomatal movement. The extreme case of the completely homobaric hypostomatous leaf in this regard is well exemplified in the experiments of Darwin (1916) on *Prunus*

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*laurocerasus*, in which the effect on porometer readings of vaselining part of the leaf was investigated.

2. Recent investigations (Williams, 1947; Heath and Williams, 1948; Heath, 1948) have shown that the behaviour of stomata under a porometer-cup, when subjected to the techniques hitherto in use, is markedly different from that of the stomata of the rest of the leaf; this discovery will undoubtedly necessitate a re-examination of the theory of the resistance porometer elaborated by Penman (1942, and Appendix to Heath, 1941), and of the results obtained by Heath (1939, 1941) in his experimental verification of the theory. In Penman's equations the parameter  $b$  fixes the boundary-condition of air-flow; this was taken by Heath as the mean distance from the centre of the cup to the nearest main veins, which for the size of leaf used was approximately 2 cm. If, however, the porometer can pull air *across* the main veins, this value will be too low.

The present author has already stated (Williams, 1948) that some slight evidence had been obtained for the movement of air across the veins; the problem has now been the subject of a specific investigation, the results of which are presented in this communication.

## INVESTIGATION

### I. Porometer Experiments

#### 1. Material and methods

The experiments were carried out on *Pelargonium zonale* var. *Paul Crampel*, plants of which were obtained from a nursery and propagated in the Bedford College Botany Garden. All measurements were taken with a resistance porometer (Gregory and Pearse, 1934), using a simple circular brass cup; the free area within the cup was a circle of approximately 0.4 cm. radius, the width of the surrounding washer approximately 0.7 cm. The leaf was illuminated by a 100-watt lamp in a water-screen; air was *pulled* through the leaf by a constant-pressure aspirator of conventional design, and the condition of abnormally wide stomatal opening within the cups (Heath and Williams, 1948) therefore obtained. The manometer reading (i.e. the quantity  $P_2$  of Gregory and Pearse, or the quantity  $P_1 - P_2$  of Heath) was used as a measure of stomatal aperture, decreasing pressure representing stomatal opening; values given in the text represent negative pressures in centimetres of water.

#### 2. Experimental results

*Experiment 1.* A porometer-cup was attached as follows: (i) the washer was lightly greased with the usual bees-wax—'vaseline' luting-wax; (ii) the lower surface of the selected leaf sector was gently pressed on to the washer, so that a wax 'marker-ring' was impressed on the lower epidermis; (iii) the upper surface of this sector was then vaselined, the area so covered extending across the boundary veins to a line approximately bisecting each adjoining

sector; (iv) the lower surface outside the marker-ring was similarly vaselined; and (v) the porometer-cup washer was then greased in the usual manner and the cup fixed as accurately as possible in the original position as determined by the marker-ring. Provided the vaselining is effective, the porometer cannot now draw air from the sector to which the cup is attached. In practice, in the case of a leaf as hairy as that of *Pelargonium*, it is probable that minute leaks through the vaseline coating remain; but more fluid substances, such as liquid paraffin, were discarded because of the possibility of injection of the leaf.

The stomata were allowed to attain equilibrium in light, the pressure then recorded being 7.86 cm. A copper-foil shield was then applied, following as closely as possible the lines of the vaselined area; this area remained fully illuminated, the rest of the leaf being darkened. After 60 minutes the stomata were once more steady at a pressure of 8.72 cm. Since the stomata under the cup have not been exposed to any change in conditions, it is reasonable to deduce that this apparent closure is due to an interference with the air-stream through the cup, brought about by the closure of the stomata in the adjoining sectors. In order to confirm that it was not due to a spontaneous closure of the stomata under the cup, possibly as a response to the abnormal treatment, the shield was removed; in a further 45 minutes the pressure had fallen to 8.20 cm. and was apparently steady. (It is possible that the failure to regain completely the original level was a damage effect, but the phenomenon is occasionally observed under apparently normal conditions.)

The shield was now replaced by one of a smaller angle, so far as possible leaving only the experimental sector illuminated. The manometer rose to the higher value of 9.44 cm.; possibly either the original shield allowed light to reach a small part of the unvaselined area, or there was a slight air-leak through the vaseline coating, or both.

The leaf was now cut with a razor along the lines of the vaseline boundaries; the effect is shown in Fig. 1, from the beginning of the record to the point (a), and it will be seen that the manometer reading has dropped sharply to the previous 'fully illuminated' value. At (a) one of the cut edges was sealed with liquid paraffin applied by means of a fine sable brush, and at (b) the other cut edge was similarly treated; this efficient blocking resulted in a considerable rise in manometer pressure. At (c) a new razor cut was made, on one side only, in the adjoining sector as close as practicable to the main vein separating it from the experimental sector; and at (d) a similar cut was made in the other adjoining sector. The slight rising tendency after the fall consequent on each of these latter operations is possibly due to the fact that the vaseline tended to penetrate the cut edges, causing a slight progressive injection of the intercellular spaces. Finally, the stomata of the lower epidermis under the cup were examined by Lloyd's method (1908) and found as expected to be wide open.

*Experiment 2.* A leaf was set up precisely as in Expt. 1, and all except the experimental sector darkened. When equilibrium had been attained, a series

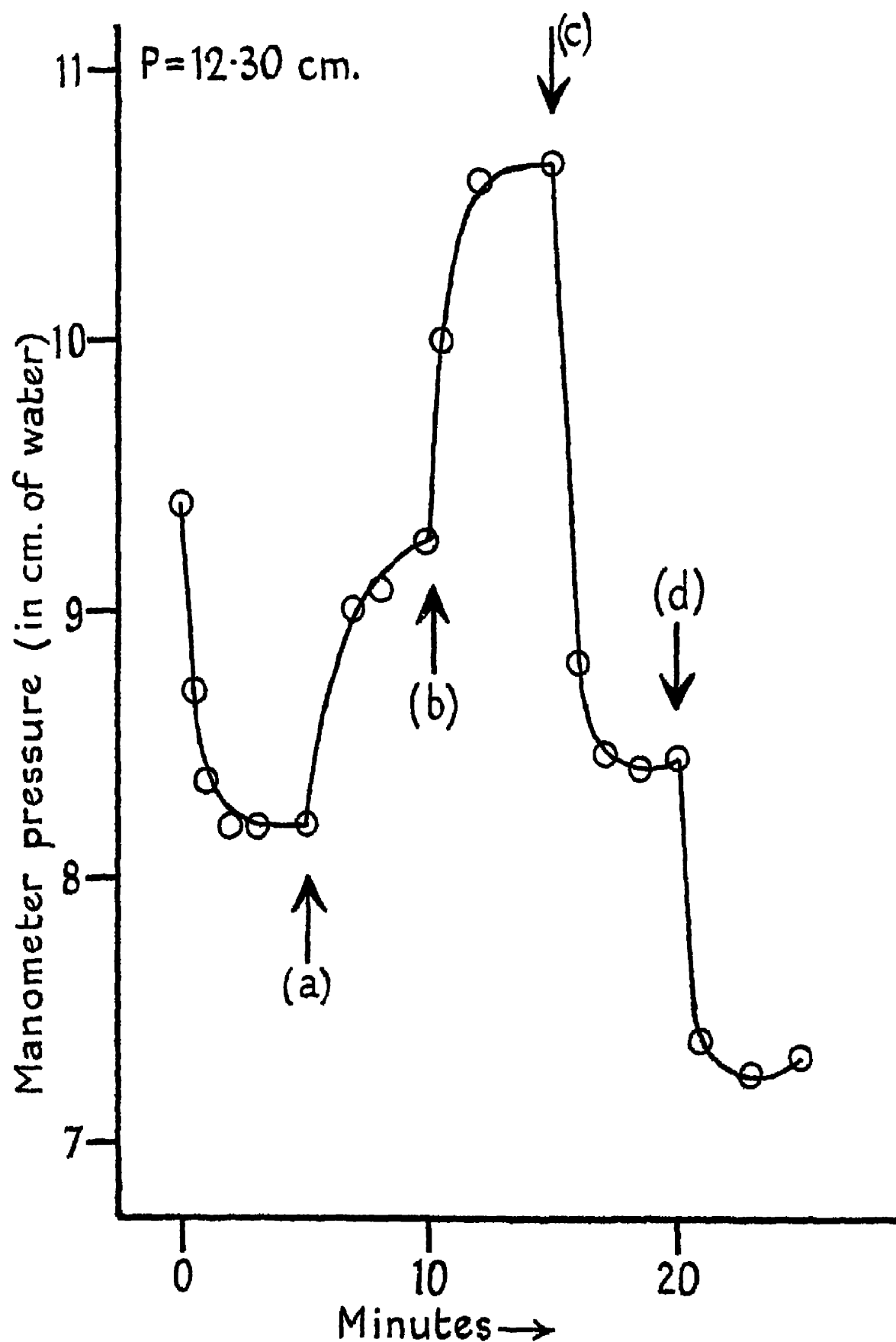


FIG. 1. Effect on porometer readings of razor cuts made in adjoining sectors.

of successive razor cuts was made in the adjoining sectors, as close beyond the boundary veins as possible. The cuts were as follows:

- (i) From the margin of the leaf to about  $\frac{1}{4}$  in. in on one side.
- (ii) Continuation of cut for a further  $\frac{1}{4}$  in. on the same side.
- (iii) A further  $\frac{1}{4}$  in.
- (iv) A further  $\frac{1}{2}$  in.; the cut now extended almost to the junction of the veins at the insertion of the lamina.
- (v) A cut of rather more than  $\frac{3}{4}$  in. in length on the other side, leaving the  $\frac{1}{4}$  in. to the margin (i.e. the region of cut (i) on the first side) undamaged.
- (vi) Cut (v) extended a further  $\frac{1}{4}$  in. towards centre; the  $\frac{1}{4}$  in. margin still uncut.
- (vii) Cut (v) extended backwards to sever the margin.

The manometer was read after each, the next cut being made when the slight rising tendency previously mentioned was observed (from 3 to 5 minutes after the cut). Table I shows the equilibrium values attained, the pressure in each case being the lowest reached before the slight rise.

TABLE I

Cut.	$P_2$ .	Drop in $P_2$ .
Start	9.60	—
(i)	8.84	0.76
(ii)	8.60	0.24
(iii)	8.56	0.04
(iv)	8.46	0.10
(v)	7.38	1.08
(vi)	7.38	0.00
(vii)	7.40	-0.02

### 3. Preliminary consideration of porometer experiments

From the results of Expt. 1 it is clear that if other available paths are blocked, air will be drawn across the main veins. Expt. 2 was designed to elucidate whether the region of communication is confined to the margin of the leaf, in which the vein is less prominent, or whether it can occur across all parts of the vein. The first cut will result in a sharp drop in pressure no matter where it is made, since it puts the intercellular spaces of the mesophyll into direct communication with air at atmospheric pressure, thus obviating the considerable resistance presented by the perforated epidermal barrier. If the channel of communication is in the marginal region only, then, in the case in which the margin is severed first, successive cuts into the sector should have negligible effect; but in the case in which it was severed last there should be an appreciable pressure drop as a result of this last cut. If, on the other hand, communication extends the whole length of the vein, air will be drawn in preference across the middle of the sector, where the cup is situated. In this case, if the margin is severed first, successive cuts will still have an effect

until the region opposite the cup is passed; but if the middle region is severed first, later cuts (including that through the margin) will have practically no effect. The experiment shows clearly that the second alternative is realized; and we may therefore deduce that adjacent sectors are in communication along the whole length of the separating vein. It is now necessary to ascertain whether the anatomical structure of the leaf is in accordance with this conclusion.

## II. *Anatomical Investigation*

Heath (1941) has observed that there are considerable intercellular spaces in the mesophyll under the small veins, and a cursory examination of the extreme margin of the leaf shows a similar situation at the ends of the main veins; these channels are not in dispute, and investigation has therefore been confined to the structure of the main veins at about the middle of the leaf. For reasons that will be clear later, sections (transverse, longitudinal, and surface) were cut by hand from fresh material; the razor was moistened with alcohol to facilitate the actual cutting, and the sections were immediately transferred to, and examined in, water. One advantage of this method is that, unless the section is too thin, air is trapped in the intercellular spaces, the extent of which is then clearly seen by means of the striking optical effects which result.

A diagram of a vein in cross-section is given in Fig. 2 (a). It may be said immediately that there seems to be no practical possibility of lateral air movement across the tissue on the *ventral* side of the bundle; there is an extensive system of intercellular spaces running longitudinally, but lateral connexions could only rarely be observed. The structure of the region dorsal to the bundle is very different, and is best considered in relation to the structure of the lamina. The leaves examined by the writer were rather more robust than those studied by Heath (1941); the lamina was usually 200 to 250 $\mu$  thick (instead of 180 $\mu$ ), and of this thickness the palisade occupied approximately 80 $\mu$  (instead of 36 $\mu$ ). This discrepancy is to be expected, since the writer's plants were grown in full sunlight, whereas Heath's plants were designedly grown in shade to obtain maximum area of lamina. The palisade seems regularly to consist of two layers which, though reasonably clearly differentiated from the spongy mesophyll, consist of cells rather less elongated (barely twice as long as broad in section) than might be expected; *and these two layers are continuous over the dorsal surface of the vein*. Immediately over the vein, however, the cells are almost isodiametric, less regularly arranged, and less closely packed; the appearance is rather of somewhat compact spongy mesophyll than of palisade, and the total thickness of the layers is in consequence somewhat reduced (about 55 $\mu$ ) in this region. Chloroplasts are fairly numerous in the cells, though less so than in the palisade of the lamina. In sections of fresh material mounted in water these layers are seen very clearly on account of their green colour and extensive air spaces; but in microtome sections of material prepared by the normal paraffin method they are

not easily distinguished from undifferentiated parenchyma, and it is for this reason that this method was discarded. A camera-lucida drawing of the region in question as seen in cross-section is given in Fig. 2 (b).

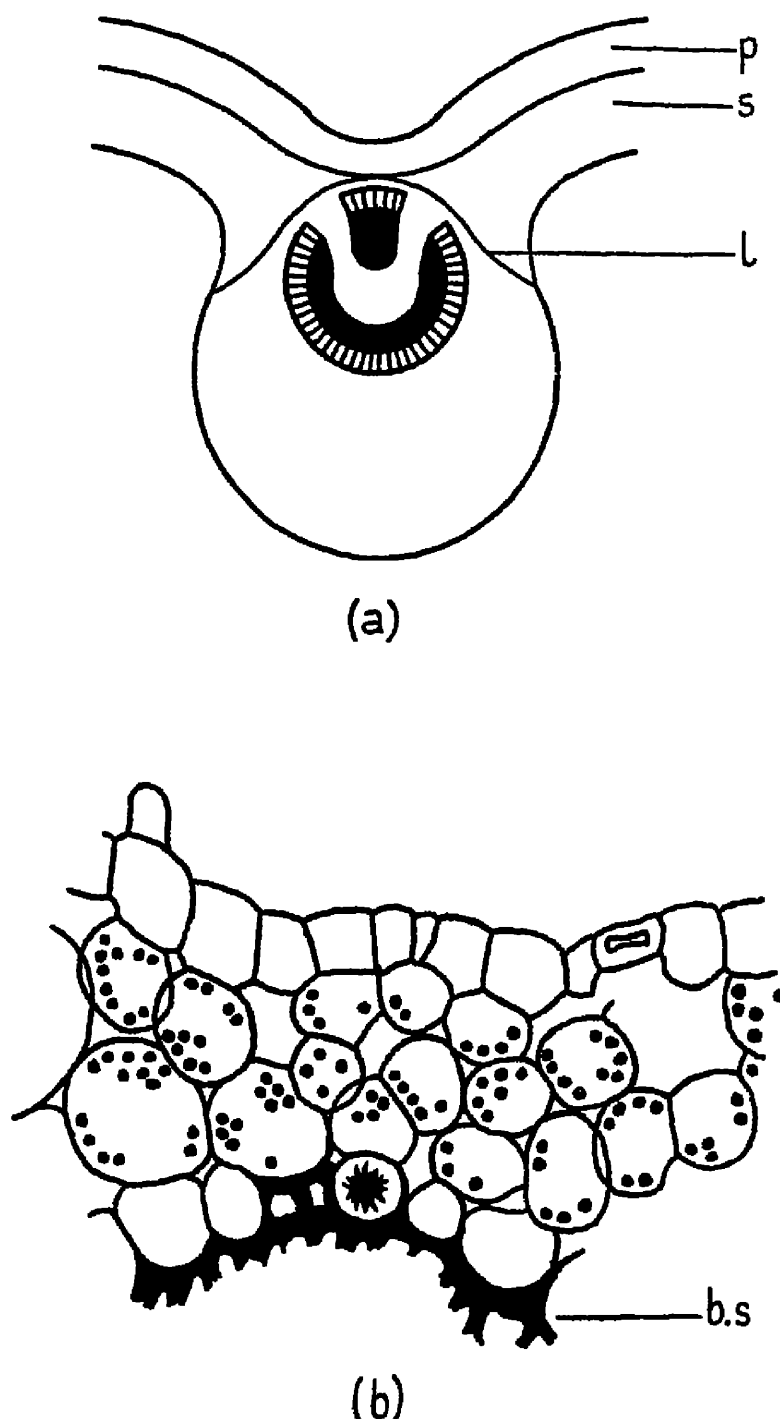


FIG. 2. Structure of main vein of leaf of *Pelargonium zonale*. (a) Diagrammatic representation of arrangement of relevant tissues. Xylem black, phloem shaded; *p*, palisade mesophyll; *s*, spongy mesophyll; *l*, approximate line of demarcation of spongy mesophyll from parenchymatous ground-tissue of vein. (b) Camera-lucida drawing of region immediately dorsal to vascular bundle. *b.s*, beginning of thick-walled sheath of vascular bundle.

Surface sections show that stomata are present in the upper epidermis over the vein. They seem rarely to occur immediately over the centre of the vascular bundle, though they have been seen; but the distribution of the stomata in the upper epidermis is so irregular and so variable that it is doubtful if this observation has any significance.

It is thus clear that the anatomical findings are in complete agreement with the predictions made from the results of the porometer experiments.

## DISCUSSION

### 1. General considerations

It is clear from the preceding sections that the intercellular space system of the leaf of *Pelargonium zonale* is continuous throughout the leaf, communication between sectors being established via a few layers of mesophyll on the dorsal side of the veins. However, Heath (1941) has shown conclusively that when the stomata are wide open the greater part of the porometer air-stream is drawn in through the stomata immediately over the cup and passes *vertically* through the leaf; but that with increasing closure that part of the air drawn *laterally* through the mesophyll is of increasing importance. If the stomata are fully open air is therefore unlikely to be drawn across the veins unless the upper surface has been blocked, either by vaseline as in the experiments here described, or by enclosure in any form of porometer system not at all times open to the air (e.g. in leaf-chambers such as used by Heath, 1939 and 1941). However, this is true only of the very wide apertures now known to be attained only under a porometer-cup or in CO<sub>2</sub>-free air (Heath and Williams, 1948; Heath, 1948). Such apertures are not attained on a leaf freely exposed to the air; nor, even under a porometer-cup, can they normally be obtained during the period from about January to March, when growth of *Pelargonium* in this climate, even in a greenhouse, is at a minimum. The phenomenon of lateral air-flow across the veins is therefore more likely to be observed in experiments carried out at this time of year. We may now conveniently consider the bearing of these results on the two lines of work specifically mentioned in the Introduction.

### 2. The work of Williams (1948)

The present writer demonstrated the independence of sectors by fixing two cups, one to each half of a leaf, and illuminating and darkening the cups alternately. It is now clear that this demonstration was invalid, since the stomata under examination were either (i) fully open, in which case the air-stream was almost entirely vertical; (ii) closed, in which case there was virtually no air-stream; or (iii) moving from one of these positions to the other, in which case interaction would only affect the form of the curve obtained, and we have insufficient knowledge of stomatal dynamics to recognize such a modification. However, it is also clear that the results recorded in the paper in question are valid so long as the experiments were all conducted at or near the fully open position, which fortunately was the case. Moreover, Expt. 1 and Fig. 1 of this paper show that any interaction effect will be almost immediate, and incompatible with the delay of from 4 to 6 minutes exhibited at the cup farther from the burn used as the shock-stimulus. It is possible, however, that the phenomenon might have contributed to the slight 'initial effect' that was observed.

### 3. *The work of Heath (1939, 1941)*

It is evident that the value of 2.0 cm. taken by Heath for the parameter  $b$  in Penman's equations is too low. However, as Heath (1941, p. 474) has pointed out, even quite large errors in  $b$  can be tolerated provided that (i) the stomata are not near their closed position, and (ii)  $(b^2 - a_2^2)$  is large compared with  $a_1^2$ , where  $a_1$  and  $a_2$  are respectively the inner and outer radii of the gelatine washer. Both these restrictions may in practice prove inconvenient; the need to study the physiology of stomata at apertures of the order of those found in ordinary air is likely to focus attention on behaviour at apertures smaller than those hitherto found most convenient; and the second restriction renders small leaves, which are usually more readily available than leaves of the size used by Heath, unsuitable for use in critical work. It is not clear, however, what value of  $b$  should be taken. As Heath has pointed out, the main channel of lateral movement *within a sector* must be through the spongy mesophyll near the ventral surface of the leaf; and it seems certain from anatomical considerations that the resistance to lateral movement across the veins must be appreciably higher than that to movement across a path of the same length within a sector. As far as lateral movement of air is concerned, the leaf must be regarded as a system of substantially uniform moderate resistance, traversed by radially arranged high-resistance bars. The present writer is not competent to undertake the necessary mathematical investigation, and can only refer the new evidence to the originator of the theory for consideration.

### 4. *Conclusion*

Although this work has been concerned solely with *Pelargonium*, it is not without its general application. It is evident that a purely morphological survey of a leaf for 'vein-islets' is insufficient to establish its heterobaric nature. In any work in which the question may be of importance, it is essential to confirm, preferably both by means of the porometer and anatomically, that there exists no lateral connexion whatsoever. Failure to observe this precaution may produce puzzling discrepancies in results.

### SUMMARY

1. It is shown, both by porometer experiments and by direct anatomical observation, that the intercellular space systems of the sectors of the leaf of *Pelargonium zonale* are in communication with each other across the main veins; and that, contrary to the view hitherto held, a porometer may in certain circumstances draw air across these veins.

2. The bearing of this observation on previous work of the present author on shock-transmission, and on the work of Heath on porometer theory, is discussed.

### ACKNOWLEDGEMENTS

The writer wishes to express his indebtedness to Dr. O. V. S. Heath for the privilege of a number of stimulating discussions on porometer theory in general, and on this small problem in particular.

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# The Conserving Influence of Oxygen on Respirable Substrate

BY

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With two Figures in the Text

## INTRODUCTION

**I**N the respiration of animal cells, and of yeast, oxygen exerts a conserving influence on organic substrate; the loss is higher under anaerobic than under aerobic conditions. This effect, which has been termed the Pasteur effect, has repeatedly been demonstrated in animal tissue by direct determinations of respirable substrate. The respiration of animal and plant cells is fundamentally similar, yet there has not yet been an unequivocal demonstration of the Pasteur effect for the cells of higher plants.

For half a century research workers have deduced from measurements of the relative rates of production of  $\text{CO}_2$  in air and in the absence of oxygen that the anaerobic loss of carbon is greater than the aerobic loss. We may recall the mass of measurements of the ' $I/N$ '<sup>1</sup> ratio given in such standard works as 'Kostytschew' (1927), or the more recent classical researches of Blackman (1928). Because the only end-product of aerobic respiration which contains carbon is  $\text{CO}_2$ , it is clear that if the  $I/N$  ratio exceeds unity, the loss of carbon must be greater under anaerobic conditions.

However, the  $I/N$  ratio for higher plants is often less than unity, and frequently very much less. When this is so, estimation of  $\text{CO}_2$  alone must leave some doubt about the occurrence of the Pasteur effect, even if assumptions about the production of another end-product (e.g. ethyl alcohol) suggest increased loss of carbon under anaerobic conditions.

It has been demonstrated that, if the production of ethyl alcohol is estimated as well as that of  $\text{CO}_2$ , under anaerobic conditions, and the assumption is made that there are no other end-products containing carbon,  $I/N$  ratios less than unity do not preclude the operation of the Pasteur effect (see Fidler, 1933). But there still remain assumptions; the demonstration is not unambiguous. This note is written primarily for teachers of plant physiology, who need, perhaps more than the research worker, clear statements of fact in their text-books. Thus Thomas (1947) writes (p. 297): 'Although suggestive hypotheses consonant with experimentally determined values of  $\text{CO}_2$  output may be put forward when  $I/N$  quotients are found to be unity or less than

<sup>1</sup> Under the old convention this meant 'intramolecular respiration' : 'normal respiration', or, as we should now say, 'anaerobic' : 'aerobic'.

unity, the present writer maintains that no conclusions about the possible conserving effects of oxygen can be legitimately drawn unless simultaneous measurements are made of the production of substances other than carbon dioxide or of the consumption of respirable substrates.'

During the years 1933 to 1935 the writer was accumulating data for the production of a balance-sheet for carbon in the aerobic and anaerobic respiration of apples. Part of these data may be used to demonstrate that the loss of respirable substrate is lower in the presence of oxygen than in its absence.

## RESULTS

The experiments on apples (and the single experiment on oranges) were carried out at constant temperatures, on large samples of fruit. Continuous records of the rate of production of  $\text{CO}_2$  were made, and determinations of the accumulation of ethyl alcohol and of the disappearance of respirable substrate were made at intervals. The methods of analysis will be described in the following papers on the quantitative relationship between the loss of respirable substrate and the accumulation of end products, and on the role of acetaldehyde in the catabolism of carbohydrate.

The data to be considered in this note are presented in Table I and in Fig. 1. Comparison of the figures for loss of carbon as  $\text{CO}_2$  shows that only those results have been included in which the  $I/N$  ratio was less than unity. For most experiments this ratio was of the order of 0.65 to 0.7, but the mean values for the Bramley's Seedling apples was 0.93.

## DISCUSSION

Before discussing the data of Table I it must first of all be stated that, so far as is known, there were no end-products of respiration other than  $\text{CO}_2$  and ethyl alcohol,<sup>1</sup> and that an analysis of the complete data shows that the amounts of carbon lost as carbohydrate plus acid and appearing in the end-products practically balanced each other. It has been shown that malic acid is lost at precisely the same rate in air and in the absence of oxygen (Fidler, 1935, 1937). It may therefore be excluded from the argument, since if included as a substrate it would merely have the effect of moving all graphs on the right-hand side of Fig. 1 upwards without altering their positions relative to each other.

Further, it is emphasized that these experiments lasted for a long enough time for the rates of production of  $\text{CO}_2$  to have reached adjusted states, in the absence of oxygen. The effects are not masked by the transitional inflections of the rate curves for  $\text{CO}_2$ -production normally observed when air is replaced by nitrogen (see Blackman, loc. cit.).

Figure 1 is constructed from the data of Table I. For each variety of fruit, the graph for loss of carbon as  $\text{CO}_2$  in air lies at a higher level than that

<sup>1</sup> Excepting acetaldehyde, which was estimated and is included in the 'alcohol number' (see Fidler, loc. cit.).

obtained in nitrogen. In the old notation ' $I/N$  is less than unity'. When zymasic products are included (central graphs), the relative positions are reversed; the loss of carbon as  $\text{CO}_2$  plus alcohol is less in the presence of oxygen. This result is in accordance with our previous observations.

TABLE I  
*The Loss of Carbon from Apples and Oranges in Air and in Nitrogen*  
(See Fig. 1.)

Loss of carbon (g./100 g. fresh weight of tissue).									
As $\text{CO}_2$ As $\text{CO}_2$ + alcohol      As carbohydrate									
Variety.	Season.	Tempera- ture ° C.	Days in gas.	In air.	In nitro- gen.	In air.	In nitro- gen.	In air.	In nitro- gen.
Sturmer Pippin Apple (English)	1933-4	20	8	0.104	0.090	0.104	0.158	0.12	0.44
			20	0.281	0.198	0.281	0.349	0.19	0.32
			30	0.404	0.287	0.404	0.506	0.45	0.52
			45	0.560	0.410	0.560	0.765	0.60	0.82
Sturmer Pippin Apple (English)	1934-5	3	31	0.147	0.124	0.147	0.254	0.02	0.00
			44	0.210	0.169	0.210	0.345	0.19	0.38
			64	0.316	0.235	0.316	0.950	0.14	0.73
			112	0.573	0.376	0.582	1.03	0.34	0.67
Sturmer Pippin Apple (English)	1934-5	12	20	0.256	0.177	0.256	0.585	0.22	0.41
			39	0.484	0.330	0.484	1.10	0.18	0.77
			53	0.659	0.43	0.659	1.20	0.41	0.76
Sturmer Pippin Apple (English)	1934-5	20	9	0.215	0.162	0.215	0.222	+0.08	0.06
			21	0.475	0.337	0.475	1.08	0.17	0.74
			35	0.780	0.544	0.780	1.94	0.60	1.4
Bramley's Seed- ling Apple	1934-5	12	4	0.046	0.046	0.051	0.119	+0.08	0.07
			8	0.091	0.086	0.101	0.208	0.29	+0.18
			12	0.133	0.115	0.145	0.260	0.06	0.35
			20	0.215	0.173	0.235	0.416	0.24	0.28
Bramley's Seed- ling Apple	1935-6	12	2	0.019	0.022	0.024	0.059	+0.07	+0.10
			4	0.038	0.044	0.038	0.101	+0.04	0.11
			6	0.057	0.063	0.057	0.153	0.06	0.04
			9	0.087	0.090	0.092	0.189	+0.03	+0.01
			12	0.115	0.115	0.120	0.246	0.06	0.12
			16	0.147	0.142	0.157	0.320	0.11	0.34
			20	0.180	0.167	0.190	0.350	0.07	0.15
			30	0.260	0.218	0.275	0.438	0.20	0.34
			40	0.333	0.265	0.359	0.537	0.45	0.49
South African Navel Orange	1935	12	7	0.055	0.041	0.059	0.100	+0.09	0.14
			15	0.116	0.075	0.118	0.198	0.04	0.02
			30	0.233	0.152	0.235	0.400	0.23	0.44

Estimation of the loss of carbon from the respirable substrate has a greater error than that of the  $\text{CO}_2$  or alcohol estimations; the right-hand side graphs for loss of carbon as substrate are irregular in form. But it is clear that the loss is less in the presence of oxygen. The mean slope of the graphs is similar to those for loss of carbon as  $\text{CO}_2$  plus alcohol.

This forms, then, an unambiguous demonstration of the Pasteur effect in tissues of the higher plants. Further, it supports the contention previously

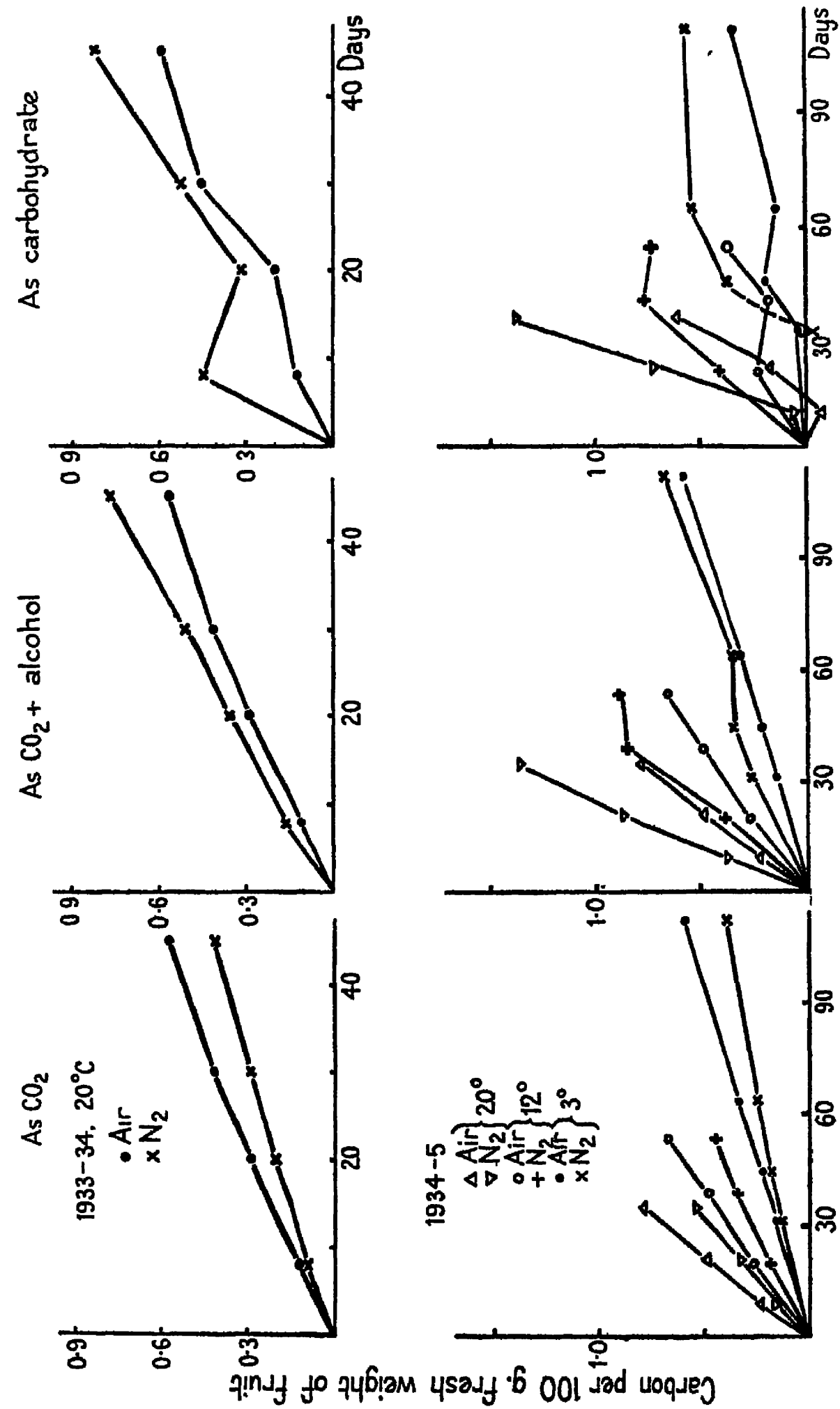


FIG. 1. The aerobic and anaerobic loss of carbon from apples (Sturmer Pippins).

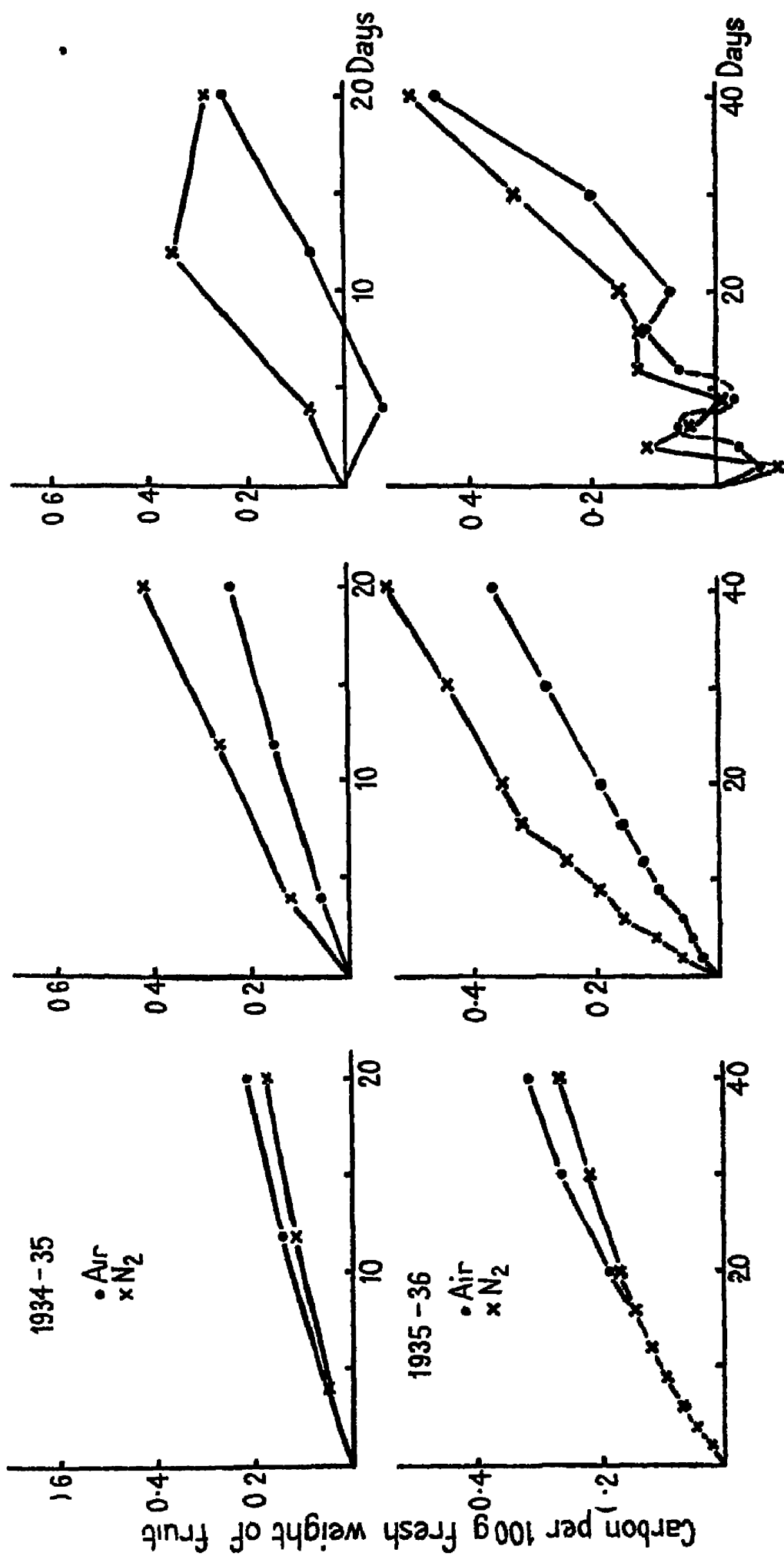


FIG. 2. The aerobic and anaerobic loss of carbon from apples (Bramley's Seedlings) at 12 C.

made (Fidler, 1933) that measurements of both the end-products of zymasic cleavage were sufficient to demonstrate this effect.

#### SUMMARY

Estimations of the loss of respirable substrate in experiments with two varieties of apple at three temperatures and in three storage seasons, and with oranges, show that the rate of loss is less in the presence of oxygen than in its absence.

This work was done at the Low Temperature Research Station, Cambridge, and was begun during tenure of the Earl Grey Memorial Fellowship of the University of Durham. It is published by permission of the Department of Scientific and Industrial Research.

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# Studies on the Morphology of Anthoceros. II<sup>1</sup>

BY

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With Plate X and nine Figures in the Text

## 3. THE DEHISCENCE OF THE CAPSULE

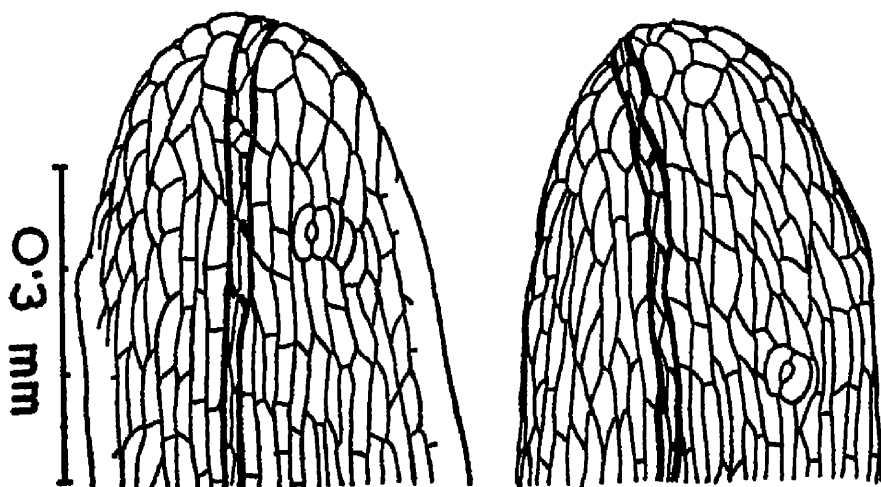
### *Introduction*

THE view expressed in most text-books and floras on the dehiscence of the Anthoceros capsule is that the wall splits progressively from the apex downwards into two equal valves, like the valves of a pod. These valves move slightly away from each other and, after it has been freed from the spore-mass, the hair-like columella projects between them. Jameson's illustrations in Macvicar (1926) only show the two separated valves, not the columella. Müller (1912-16, 1940) states that the capsule breaks open from the tip into two often spirally twisted portions and his text implies that these are free from each other apically. His figures of dehiscent capsules of *Anthoceros dichotomus* and *A. punctatus*, however, picture accurately two twisted valves cohering at their tips. Nees v. Esenbeck (1838) refers to a drawing by Corda of *A. dichotomus* with a 'curiously ropelike twisted capsule', which he considered accidental.

In 1924 Lorbeer described the dehiscence-lines of *A. laevis* briefly referred to by Campbell (1918). They run longitudinally in two depressions on opposite sides of the capsule and are continuous over the tip. Each line consists of two rows of elongate cells, although at the apex there may be up to six rows. He mentions, and illustrates by transverse sections, that the common walls between the two rows of cells are the only radial walls in the epidermis that do not become secondarily thickened, but does not mention any special thickening of the radial walls abutting on the other epidermal cells. He presumes that splitting is due to a cohesion-mechanism, and regards the presence of the two grooves as evidence of the existence of tension. He states that dehiscence is into two equal valves. Bartlett (1928), studying a number of Californian and Pacific species, recognizes several types of dehiscence. In her words: 'Although the capsules of erect sporophytes vary somewhat in the manner and extent of dehiscence, the principal steps may be illustrated by the process as it occurs in *A. pearsoni*. Tiny openings appear almost simultaneously, some distance below the tips, in the yellowish lateral sutures that are almost invariably [*sic*] present in the capsules. Once started, the

<sup>1</sup> This paper forms part of a thesis approved for the Ph.D. degree of the University of London.

slits rapidly lengthen until, on the sudden twisting of the valves, the contents of the sporogenous chambers are thrown out—the more forcibly because the twisted valves remain coherent at the tip. The latter phenomenon may be due to the drying of the small, stopper-like plugs of mucilage that exude from the tips of immature capsules on their being covered with water. These mucilaginous masses are not algal in nature, although *Rivularia* is occasionally found in them.' All the yellow-spored species (five, not including *A. laevis* nor *A. dichotomus*) examined by her show this type of dehiscence, with the exception of *A. hallii* Aust., which frequently only partially opens by one slit or even remains indehiscent 'like *Notothylas*'. On the other hand, the three



TEXT-FIG. 1. *A. laevis*. Apex of capsule with normal dehiscence-lines.

black-spored forms examined (incl. *A. fusiformis* Aust.) are described as lacking the twist in the valves, thus resembling *Megaceros* and *Dendroceros*. 'Valves flat at dehiscence' is one of her criteria for the black-spored group.

For the taxonomy of the British species discussed in the present paper and the origin of the material see Proskauer (1948).

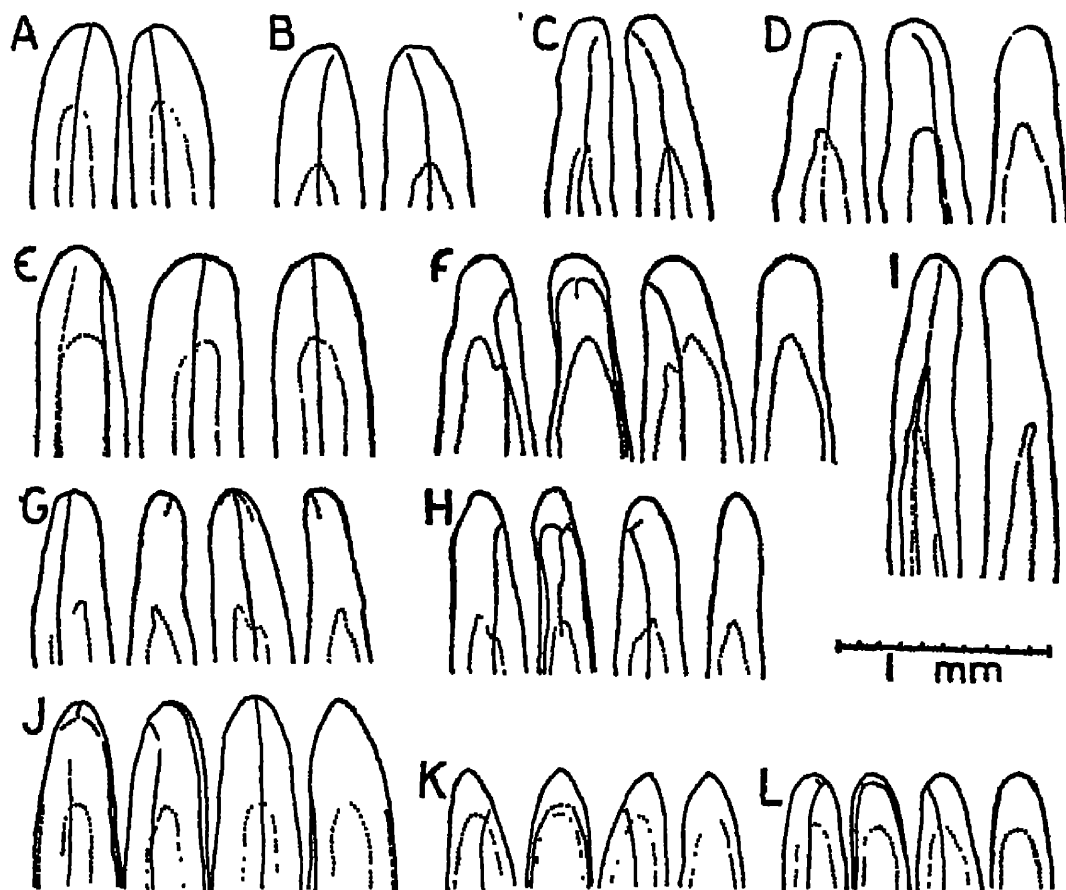
### I. The external dehiscence-lines

(a) *External features.* Sporophytes in which the tips had turned brownish were severed through the lower green region. Examination of the tips by reflected light, even when using a metallurgical microscope, proved unsuitable. The subsequent observations were, therefore, made on material from which the air was removed by suction under water with a Bunsen-pump. It was then cleared in 40 per cent. aqueous potassium hydroxide, which caused no swelling of the epidermal walls, nor changes in the shape of the spore-mass.

*A. laevis.* What seems to be the typical structure of the dehiscence-lines is shown in Text-fig. 1. The common longitudinal walls between the two rows of constituent cells are far thinner and the outer walls far thicker than in the other epidermal cells. When followed downwards into the green region, these lines become indistinct.

Commonly, however, there is some degree of variation. Among 18 tips taken from a culture, there were only 2 entirely 'normal' ones (like Text-fig. 2A).

In the abnormal tips there has either been reduction or elaboration. In the simplest case of reduction the dehiscence-lines just fail to meet at the tip (B). Further, they may become irregular (C, D, shown in detail in Text-fig. 3 A, B); thus in C, before disappearing, they are represented by single rows of cells thickened on one side only, while in D they tend to broaden before terminating in single rows. In one specimen a dehiscence-line was found on one side only and stopped short about 1 mm. below the apex.

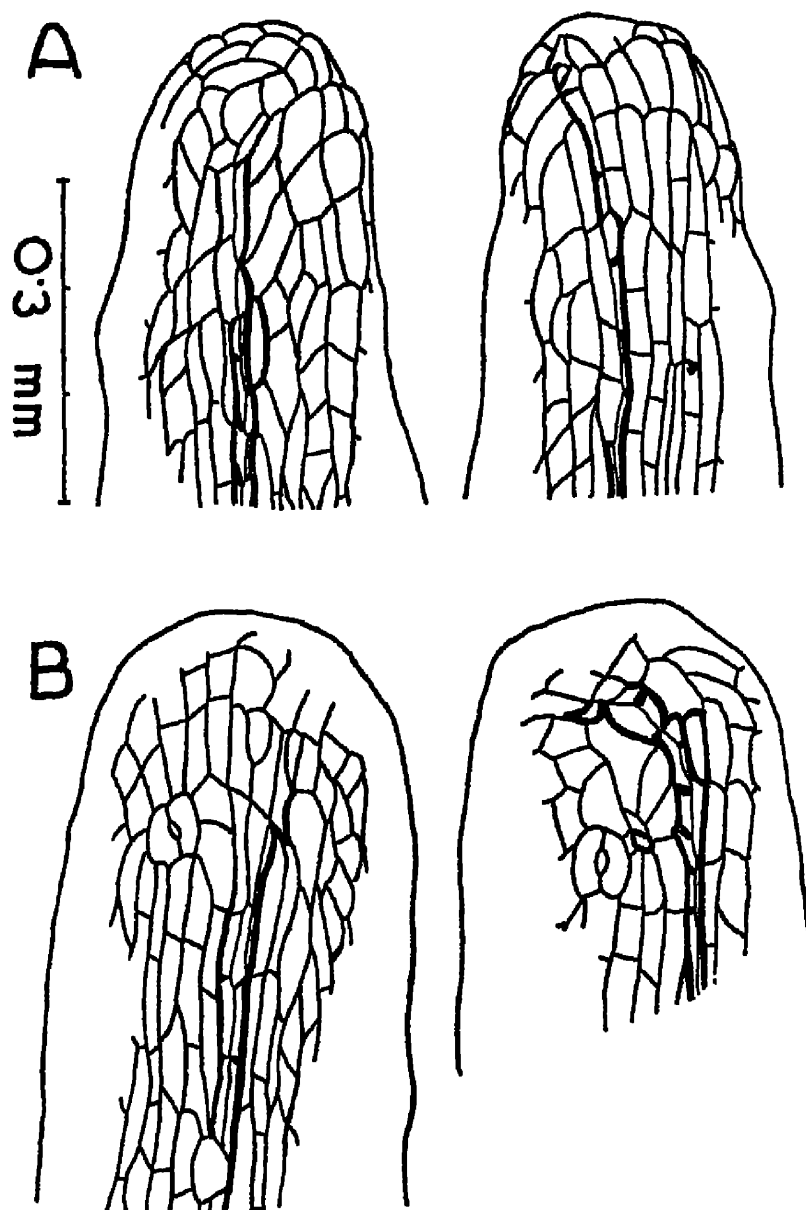


TEXT-FIG. 2. Cleared tips of capsules. A-J, *Anthoceros laevis*; K, L, *A. hurnoti*. — = ordinary external dehiscence-line. - - - = irregular external dehiscence-line. ..... = outline of spore-sac. Where more than two aspects are shown, the tip has been gradually rolled over in one direction.

Elaboration is in the form of additional dehiscence-lines. Besides the normal ones, E showed an independent, imperfect, and presumably non-functional line of the 'single-row' type (cf. Text-fig. 3 A), although usually the additional lines are connected with the main ones, in most cases at the point of junction of the latter (F). In G they are here crossed by a short hoop of non-functional lines, making four lines in all. If the point of junction were displaced towards one side of the apex, the condition shown by H (details in Text-fig. 4) and I would be reached. In one extreme case (J) three normal lines were traceable down to the green region, whereas the fourth 'single-row' line disappeared higher up.

Such variations were observed in material from different localities, whether wild or grown in culture.

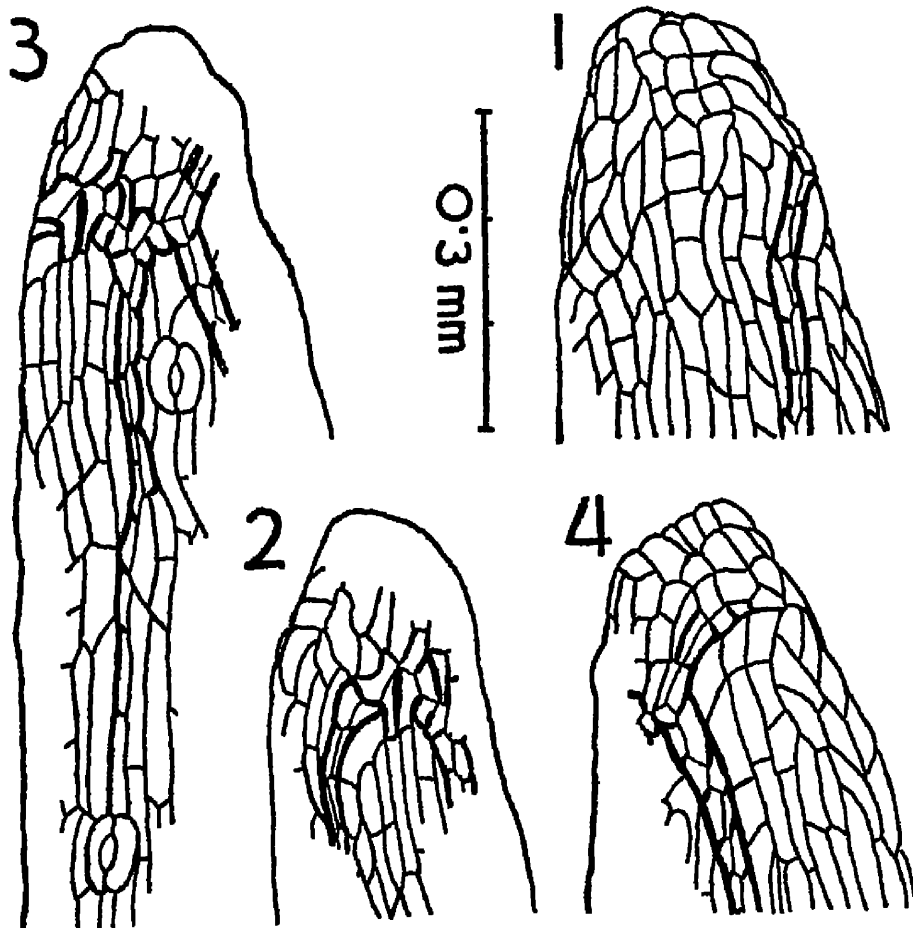
*Black-spored forms.* The general structure of the dehiscence-lines of *A. punctatus* and *A. husnoti* is like that of *A. laevis*. A somewhat higher proportion of tips are normal, but there is the same range of variation from slightly abnormal tips (Text-fig. 2 K, L) to such as show up to four dehiscence-lines near the apex.



TEXT-FIG. 3. *A. laevis*. Tips of capsules with incomplete dehiscence-lines. (A = Text-fig. 2 c; B = Text-fig. 2 d.)

(b) *Histology.* A transverse section through the maturing region of a typical sporophyte of *Anthoceros* shows the two external dehiscence-lines lying in depressions on opposite sides of the capsule-wall. In *A. laevis* (Pl. X c), as opposed to the black-spored forms (Pl. X d, *A. husnoti*), the capsule is usually markedly compressed in a plane at right angles to that containing these lines. The depressions corresponding to them are present already in the embryonic region (A). If the cells constituting the dehiscence-line are examined in progressively more mature regions of capsules (Pl. X B-H, *A. laevis*), the common walls between the two rows are seen to lack the additional thickening layers laid down on the walls of the other epidermal

cells during differentiation, while the outer walls and those abutting on adjacent epidermal cells or other cells of the same row exhibit this thickening more strongly (cf. also Text-fig. 1). The thickening may even ultimately extend on to the inner walls so as to form a U-shaped hoop (H). The thickened walls of the cells of the dehiscence-line assume the dark coloration characteristic of the mature epidermal walls at a much earlier stage (i.e. at a much lower level) than the latter.



TEXT-FIG. 4. *A. laevis*. Tip of capsule with additional dehiscence-lines (cf. Text-fig. 2 H).

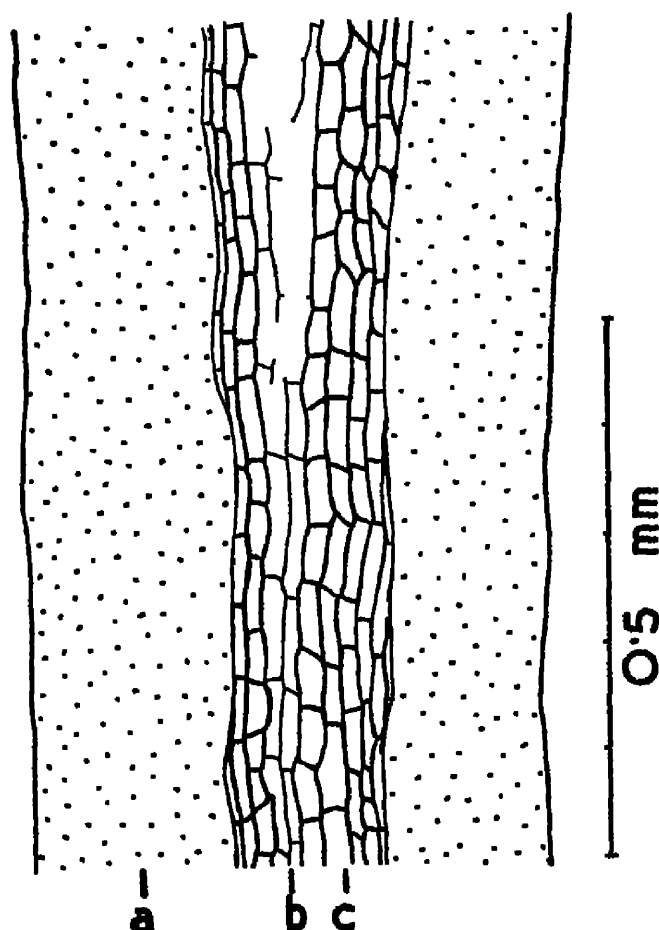
## II. The internal dehiscence-lines

In transverse sections of mature regions of sporophytes, especially of *A. laevis*, there is recognizable a special differentiation of the innermost layer of the wall (Pl. X c; cf. Cesares-Gil, 1919), which may be spoken of as the lining-layer. In appearance it is reminiscent of a thickened endodermis. The inner walls, abutting on the spore-sac, and the radial walls of its cells are indurated and ultimately show the same dark coloration as the pseudo-elaters, the outer spore-coats, and the thickening layers on the epidermal cells. The thickening is, however, absent along two strips, usually two cells wide, situated opposite the two external dehiscence-lines (Pl. X c, g). In transverse section there are thus two hoops of thickened cells on the inside of the wall. Text-fig. 5 shows a part of the lining-layer with the rows of unthickened cells as seen from the inner surface. In the upper part of the figure the unthickened cells have broken down owing to dehiscence, so that

the hoops of thickened cells have separated. The strip of unthickened cells can be considered as an internal dehiscence-line.

### III. The spore-sac

In the drawings of cleared tips of capsules (Text-fig. 2) the limits of the spore-sac are indicated by dotted lines. The mass of mature spores terminates some distance from the actual apex, usually approaching it more closely in



TEXT-FIG. 5. *A. laevis*. Capsule (mature region) split in a tangential longitudinal plane at right angles to that containing the dehiscence-lines. Seen from cut surface. *a* = chlorenchyma; *b* = internal dehiscence-lines; *c* = thickened cells of lining-layer.

black-spored forms than in *A. laevis* (cf. the longitudinal section through a capsule tip of *A. laevis* shown in Pl. X I). Pl. X J shows a transverse section through the sterile tip of a capsule of *A. husnoti*, the external dehiscence-lines being marked by arrows. It is noteworthy that the irregularities in the external dehiscence-lines are for the most part restricted to this sterile region.

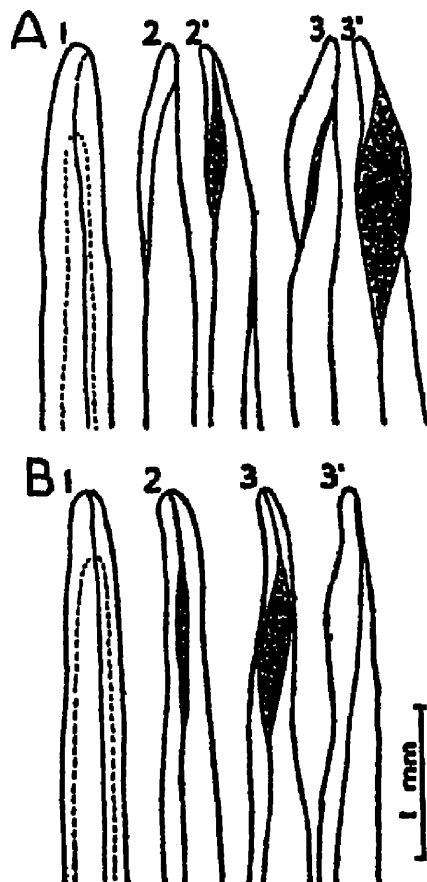
### IV. Dehiscence

When the tip has assumed the brown or black coloration characteristic of the respective species, the capsule is ready for dehiscence, a process that depends on water-loss. The tip of the maturing capsule of *A. laevis* shown in Text-fig. 6 A1, after being kept under fairly moist conditions for a week, dehiscd when examined by direct illumination under the microscope.

As moisture is lost to the atmosphere, the tip gradually shrivels. As it shrinks, the wall meets with the resistance of the hard mass of spores

and elaters in the fertile region. It is at this level that a slight split first appears in one of the dehiscence-lines. This split gradually widens and enlarges downwards, sometimes also extending upwards for a small distance into the sterile region (Text-fig. 6, A<sub>2</sub> and A<sub>2</sub>', tips drawn from opposite sides).

As soon as the internal mass is exposed, the pseudo-elaters can be seen performing twisting movements among the spores. It is, therefore, reasonable to assume that, during the drying process, these hygroscopic structures



TEXT-FIG. 6. *A. laevis*. Dehiscence of capsule (see text). Limit of spore-sac indicated by broken line.

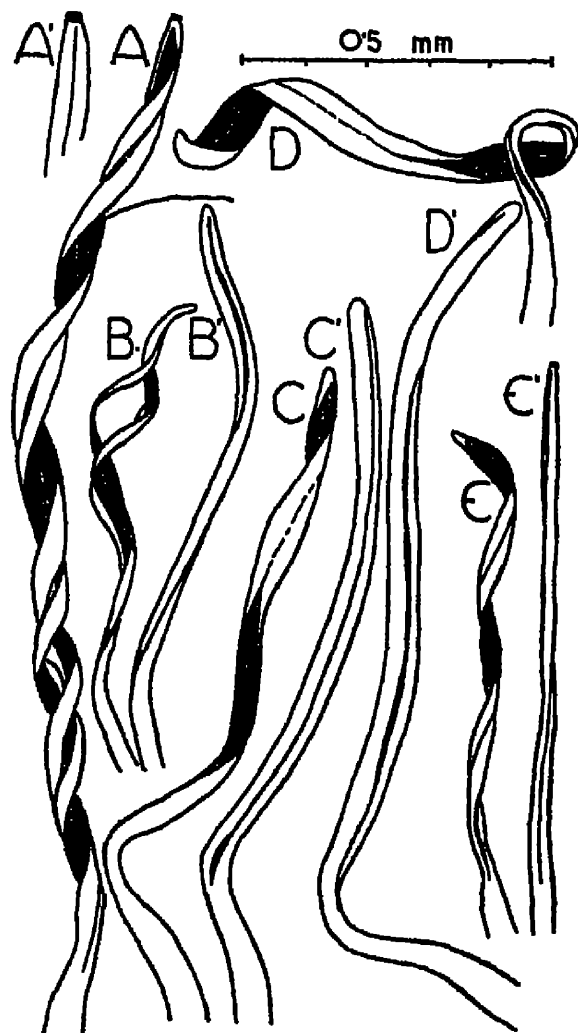
are under tension. This would result in an active back-pressure of the spore-mass on the jacket. Both epidermis and lining-layer are capable of hygroscopic movement (see § VI). In sections through mature tips the jacket tissue between the external and internal dehiscence-lines has mostly broken down, so that a rupture of the dehiscence-lines has alone to be effected (Pl. X C, H). The pressure of the shrinking jacket against the spore-mass and the hygroscopic movements of the pseudo-elaters and indurated jacket-layers will supply the necessary force.

Splitting may now take place also along the other dehiscence-line (A<sub>3</sub>), although this one (B<sub>3</sub>') sometimes remains intact. Even where two splits occur, they do not normally extend to the apex of the capsule, nor do they originate in the sterile region. As previously pointed out, the irregularities in the dehiscence-line structure are mostly confined to this region, so that they play no part in the process of dehiscence. Where more than two perfect

dehiscence-lines are present in the fertile region (Text-fig. 2 j) several splits could occur, but dehiscence by more than two valves has not been observed.

#### V. *The behaviour of the valves in the dehisced capsule*

In undisturbed capsules, growing under natural or cultural conditions, splitting along the dehiscence-lines generally appears to stop short of the



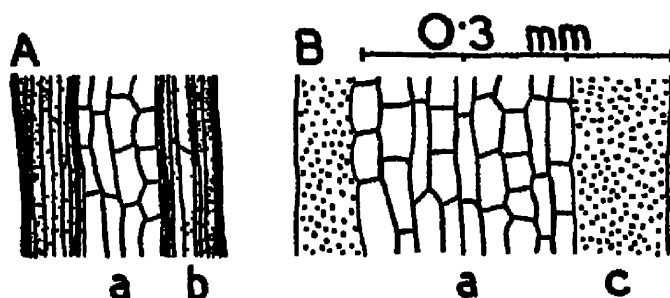
TEXT-FIG. 7. *A. laevis*. Various methods of dehiscence of capsule. In each case the same capsule is shown dry and moist. Columella indicated by line, spore-elater mass omitted. Calyptra in black in A and E. (Cf. also Pl. VIA, Proskauer, 1948.)

actual apex. The valves, which progressively become detached downwards as ripening proceeds, become spirally twisted as they dry (Text-fig. 6 A). This twisting is a reversible hygroscopic reaction, although the valves do not respond to slight changes in atmospheric humidity (e.g. those caused by breathing) such as will induce strong movements in other hygroscopic structures like the peristome-teeth of many mosses. In a very humid atmosphere, however, and especially on wetting, the valves almost completely untwist and close the sporogonium. Text-fig. 7 B shows the open valves in a capsule of *A. laevis*, while B' shows the condition after soaking in water. If such a soaked tip be placed on a heated slide, drying and twisting takes place. Twisting and untwisting can be brought about in this way more or less indefinitely.

Dehiscence frequently takes place by a single split only (Text-fig. 7 B). Occasionally, one dehiscence-line splits at one, the other at another level (c, D), the valves in such cases still showing the twist. By repeated wetting and drying of the tip shown in B, the second dehiscence-line was ultimately caused to break open.

Fig. 7 also illustrates the variation in capsule thickness which is found in all species. The 'calyptra', shown black, is still present in A (cf. Proskauer, 1948). It swells up considerably on moistening.

Some spores will be shed from the open capsule by the hygroscopic movements of the valves and the more sensitive pseudo-elaters and columella. If cultures with dehisced sporophytes are lifted from garden-frames into



TEXT-FIG. 8. *A. laevis*. Portion of a valve of a capsule. Mounted in water. A, rolled up (seen from concave side); B, ditto, flattened out. a = lining-layer; b = epidermis; c = broken chlorenchyma.

the open, showers of spores fall from the open valves to which they had adhered. Air-currents probably play an important part in dissemination of the spores.

All the above observations apply equally to *A. laevis* and the black-spored species *A. husnoti* and *A. punctatus*. In the monocarpic *A. punctatus* the gametophytes die before spore-production and -liberation is completed (cf. Proskauer, 1948). As the thalli decay, partially dehisced or indehisced capsules frequently drop to the surface of the soil or culture medium where spore-liberation takes place by rapid decay of their softer tissues.

## VI. The hygroscopic mechanism

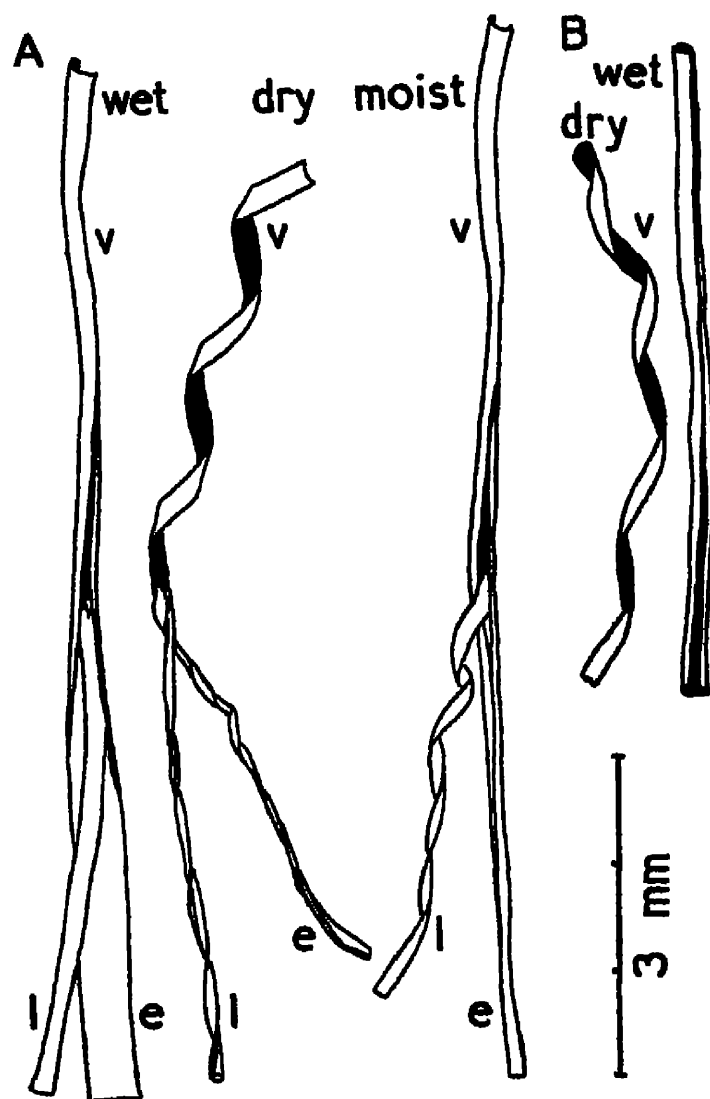
In the numerous dehisced capsules observed of plants in nature and in culture the twisting of the valves is invariably in the direction of a 'right-handed' spiral. This is true of entire valves, as well as of isolated pieces of valves. An attempt was made to investigate the nature of the motor-tissues involved.

A dried twisted piece of valve shows in section a shallow curvature, the convex side corresponding to the original outer surface (Text-fig. 9 B). On moistening, swelling occurs and the curvature becomes greatly increased; the two edges of the valve may meet and even overlap.

The epidermis and lining-layer, the only thickened tissues, occupy the outer and inner surfaces respectively of the valve, and of these the epidermis is the stronger. They are separated by the remains of the chlorenchyma

(Pl. X c). Text-fig. 8 A shows a piece of a rather narrow valve of *A. laevis* wetted and rolled up, and seen from the concave side. In B, it has been flattened out under a cover-slip, the lining-layer now lying exposed as a strip of large cells with thickened walls.

After flattening a soaked valve it is possible with fine needles to dissect the lining-layer away from the epidermis, to which the remains of the chloren-



TEXT-FIG. 9. *A. laevis*. Hygroscopic motor-tissues of capsule (see text). *e* = epidermis and chlorenchyma; *l* = lining-layer; *v* = entire piece of valve.

chyma adhere. Using a piece of valve of *A. laevis*, this separation was performed to about one-half, the remainder being left intact (Text-fig. 9 A, wet). The ends of the epidermal strip were allowed to rest on the extended tips of a pair of fine forceps, whilst the free lining-layer was left dangling. Drying was accomplished by holding the whole slightly above a heated slide. The lining-layer is thus prevented from becoming entangled with the remainder of the strip as twisting occurs and also from adhering to the slide. The figure shows that the intact piece of valve exhibits the characteristic open spiral, whereas the separated parts each show a close spiral twist in the same direction, especially obvious when the specimen had been slightly remoistened (Text-fig. 9 A, moist).

*A. punctatus* and *A. husnoti* are precisely similar as far as the general structure and behaviour of the valves are concerned. Twisting of the (less indurated) lining-layer after separation tends here, however, to be somewhat irregular; in one preparation of a valve of *A. husnoti* the lining-layer even exhibited turns of a 'left-handed' spiral, i.e. opposite to those of the epidermis and of the entire valve.

As no asymmetry is apparent in the motor-tissues (epidermis and lining-layer) nor in their individual cells when examined under the microscope, it would seem that twisting, as is presumably the case with the pseudo-elaters and columella, is due to the arrangement of the units of the wall-material.

The epidermis is clearly the main motor-tissue, but the action of the lining-layer changes its twist from a close to a wider spiral.

#### VII. Observations on American species

Two of Bartlett's (1928) selected types were investigated on herbarium-material, viz.:

- (a) *Anthoceros pearsoni* Howe. (Yellow spores.) 'Moist bank, Mill Valley, Marin Co., California. May 7. 1892. M.A.H. (Type duplicate).'

The material showed the twisted valves cohering at the tips as described by Bartlett. Hygroscopic movement in these 54-year-old specimens was unimpaired and agreed altogether with that of the British species. The lining-layer agrees with that of *A. laevis*.

- (b) *Anthoceros fusiformis* Austin. (Black spores.) (1) 'On roadside bank, Mill Valley, Marin Co., California. Apr. 26.93. M. A. Howe.' (2) 'On moist soil. McCleary Pk., Portland, Oregon. Jan. 1.07. A. S. Foster.'

The valves cohere at the tips and twist precisely as in all other species examined. No lining-layer could be detected and, while this may possibly be due to bad preservation, it is more likely that the induration of its cells is very slight or lacking (cf. with the British black-spored forms).

#### Discussion

The method of dehiscence of *Anthoceros* is unique amongst bryophytes, with the exception of certain other members of its own family. Yet most of its features are to be found individually in other genera of the group. Thus a thickening of the cells of the lining-layer of the capsule wall is a characteristic of most Jungermanniales, though the wall here frequently consists of only two layers of cells. In *Calypogeia* (Macvicar, 1926) the four rather long valves acquire a spiral twist.

The not infrequent occurrence of capsules with indications of four dehiscence-lines in *Anthoceros* may point to a possible relation to the typical condition found in Jungermanniales and in *Andreaea*. In view of the incomplete terminal separation of the valves in *Anthoceros* it is of interest to consider

the dehiscence of *Andreaea*. Whereas most species dehisce by four longitudinal slits, some have more than four, and in *A. wilsonii* the valves are free apically (Brotherhus, 1924). Dehiscence by slits is, however, not restricted to Anthocerotales and *Andreaea*, since it occurs in *Pallavicinia* and its allies (Mavicar, 1926; Goebel, 1930), where there are frequently but two valves. Dehiscence by a single slit, so often seen in *Anthoceros*, is characteristic of *Haplomitrium* (Macvicar, 1926), *Monoclea*, and a Brazilian *Pallavicinia* (Goebel, 1930).

The probable importance of spore-liberation by decay in the small *Anthoceros punctatus* suggests comparison with some of the cleistocarpic mosses which also grow on arable land.

*Notothylas* resembles its ally *Anthoceros* in exhibiting a range of methods of spore-liberation. Capsules of *N. orbicularis* dehisce by two to four valves (Müller, 1912-16); those of *N. levieri* have two valves cohering at the tip, but usually open by a single suture only, and show hygroscopic twisting (Pande, 1934). Bartlett (1928), finally, speaks of indehiscence.

The customary misinterpretation of the method of dehiscence is possibly due to insufficient observations on living material. Separated valves are occasionally found on plants in the field, but these, when dry, will of course show the spiral twist. Such separation in most cases probably results from disturbance by animals or falling fragments of soil. Separation occurs rather more readily in the black-spored forms. In herbarium-material (cf. § VII and also the description of the type-material of *Anthoceros laevis* in Proskauer, 1948) some specimens at least will still show the natural condition and all will show the twist. Such material is, however, mostly examined after soaking, when the valves would be straight and, without knowledge of living plants, those with free valves would most likely be considered the normal ones. The same applied to specimens preserved in liquid media.

The forms investigated by me did not show the violent spore-ejection described by Bartlett for *A. pearsoni*, nor have I observed the 'stopper-like plugs of mucilage that exude from the tips of immature capsules on their being covered with water'. It is possible that Bartlett's plugs were merely the swelling calyptrae (cf. § V). Her assertion (see Introduction) that black-spored forms lack the twisting of the valves appears incorrect (§§ VI and VII).

It might be of interest to investigate whether the lining-layer fulfils any other physiological function apart from its role in dehiscence. As pointed out in § II, it resembles a thickened endodermis interrupted at two points by the 'passage-cells' of the internal dehiscence-lines and separating the photosynthetic jacket from the sporogenous tissue. It reaches its fullest differentiation, however, at a level at which the spores are practically mature and presumably no longer in need of nutriment. After dehiscence it covers the newly exposed inner surface of the jacket like an epidermis. This might be of importance near the lower limit of dehiscence where the jacket-cells are in part still living.

### Summary

1. The typical structure and irregularities of the external dehiscence-lines are described.
2. An account of the lining-layer and internal dehiscence-lines is given.
3. The process of dehiscence is described and its causes are discussed.
4. The hygroscopic twisting of the valves was investigated.
5. Herbarium-material of certain American species was examined on these points.
6. The dehiscence of *Anthoceros* is compared with that of other bryophytes.

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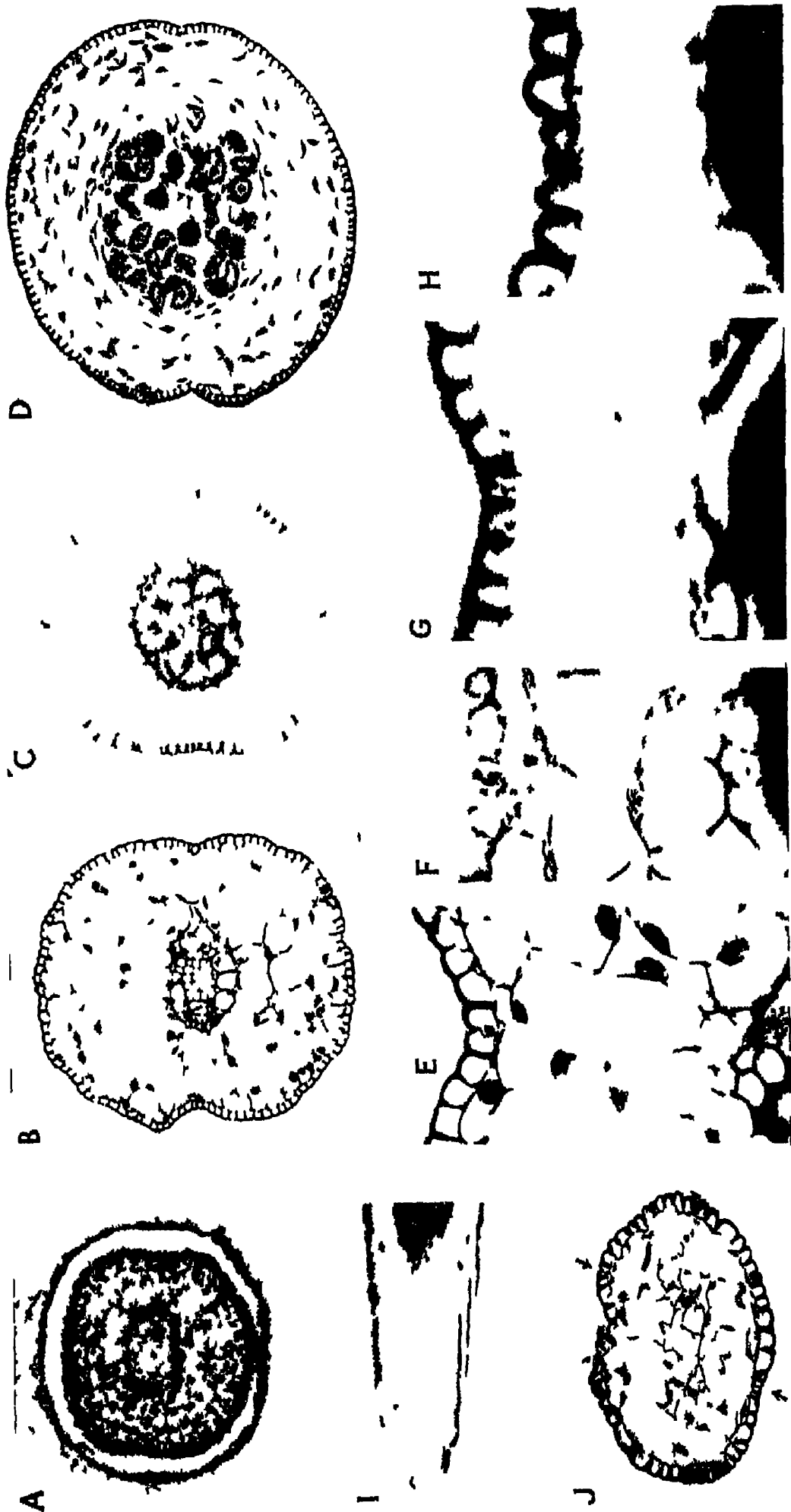
### EXPLANATION OF PLATE X

Illustrating J. Proskauer's paper on *Anthoceros*

Photomicrographs of microtome-sections of capsules of *Anthoceros*. D, J, *A. humoti*; the rest, *A. laevis*.

A, Meristematic region. B, Region of mature spore-mother-cells. C, D, Mature region. E-II, Progressively older stages in development of dehiscence-lines. I, Longitudinal section through tip of capsule; sterile tissue partially broken down. J, Transverse section through tip; the arrows mark the external dehiscence-lines. (A-C, J  $\times 120$ ; D  $\times 90$ ; E-H  $\times 475$ ; I  $\times 70$ .)





PROSKAUER—ANTHOCEROS



# The Effects of Ploidy upon the Leaf of *Musa*

BY

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With four Figures in the Text

## I. INTRODUCTION

**D**URING the development of a single banana sucker a considerable number of progressively larger leaves is produced and their persistent sheaths constitute the apparent stem or trunk of the banana plant—the pseudostem. The development and morphology of the leaf of *Musa* has been studied by Skutch (1927, 1930, and 1930a), who found that the leaf is fully differentiated by the time it is thrust clear of the pseudostem. Each lamina-half has an independent origin as an outgrowth from the midrib, and differentiation is basipetal, from the apex downwards and from the margin inwards. Vernation is convolute, the right half rolled upon itself and the left half rolled around the right half and the midrib. The true stem or axis remains very short until the whole shoot has attained its maximal vegetative development, about which time it is transformed into an inflorescence. The internodes below the inflorescence then elongate and, concurrently, the leaves they bear are reduced in size and are finally replaced by the bracts that subtend the inflorescence or bunch. An interval of three or more months may elapse between floral initiation and the appearance of the bunch from the pseudostem.

This paper is concerned with the effects of ploidy upon the leaf of *Musa*, special attention being paid to ontogenetic relationships.

## 2. MATERIALS AND METHODS

The collection of species and varieties of *Musa* maintained at this Institution, together with a range of diploids and polyploids of experimental origin, were available for study. Two seeded diploid clones ( $2n = 22$ ), representatives of the two principal species of Eu-*Musa*, have been much used. They are: *M. acuminata* Colla, Clone A (Selangor) (I.R. 53), and *M. Balbisiana* Colla, Clone Ceylon (I.R. 100), upon both of which species taxonomic comment has been made by Cheesman (1948). A number of other seeded diploids were also used (with both 20 and 22 chromosomes). Of the triploids, a few were of experimental origin, but the majority were established edible clones with  $2n = 33$  (Cheesman and Larter, 1935; Cheesman and Dodds, 1942). All the tetraploids ( $2n = 44$ ) were of experimental origin, having derived from the pollination of triploids (Cheesman and Dodds, 1942). The few mature pentaploids

available have been produced experimentally from various interspecific crosses (Dodds and Pittendrigh, 1946; Dodds and Simmonds, 1946, 1947).

*Methods.* Two main series of samples were taken. In the first (series A), the length, breadth, area, and a complete picture of the variation in thickness of the broader lamina-half of each leaf were obtained. Climax leaves<sup>1</sup> were sampled, so far as possible, from well-developed flowering stems. Areas were measured by plotting on graph-paper and counting squares. Strips of the lamina-half were torn out at intervals of 20 cm., starting at 10 cm. from the base; measurements of thickness were made at the inmost edge of the strip (next to the pulvinar band; Skutch, 1930a) and at intervals of 5 cm. outwards across it. In certain cases, when the leaves were very small or when especially detailed information was required, smaller intervals across the lamina and along the leaf were taken. Thickness was measured between the veins with a micrometer calliper (Starret, 216 M) reading to 10 $\mu$ . Care was taken to check zero reading of the instrument at frequent intervals as it was found that dirt and wax from the leaf could readily clog the surfaces of the anvil and spindle. The instrument was calibrated by taking thirty measurements of leaf material of varying thickness and measuring corresponding hand sections under the microscope. The data gave a good straight line for which

$$r = 0.9963 \text{ (} P \text{ very small),}$$

$$\text{and } b = 0.9972 \pm 0.01616.$$

The deviation from  $b = 1$  is less than the standard error, and testing whether the line may be considered to pass through the origin,

$$t [27] = 1.16, P = 0.2-0.3.$$

The calliper therefore gave an accurate direct measure of leaf thickness as judged by sectioning.

In the second series of samples (B), using central climax leaves and a collection of diploids and polyploids comparable but not identical with that studied in series A, the following measurements were made: the difference in height between the base and apex of the lamina, with the leaf in its natural posture; the length of the lamina and the breadths of the two lamina-halves; the fresh weight of the 30-cm. length of petiole-midrib that extended an equal distance above and below the base of the lamina; and the dry weight of the same piece of petiole after oven-drying for 24 hours at 100° C.

### 3. RESULTS

#### 1. *Variation in thickness within the leaf*

In Fig. 1 are recorded systematic measurements of leaf thickness for an entire climax leaf of I.R. 100, from which it is seen that maximal laminar

<sup>1</sup> In this account 'climax leaf' is applied to one of the largest leaves produced by a pseudostem before the decline in leaf size that precedes the production of a bunch. The middle leaf in the series of climax leaves borne by a single fully grown pseudostem is referred to as the 'central climax leaf'. 'Right' and 'left' are applied as judged by an observer facing the upper surface of the leaf with the petiole pointing downwards.

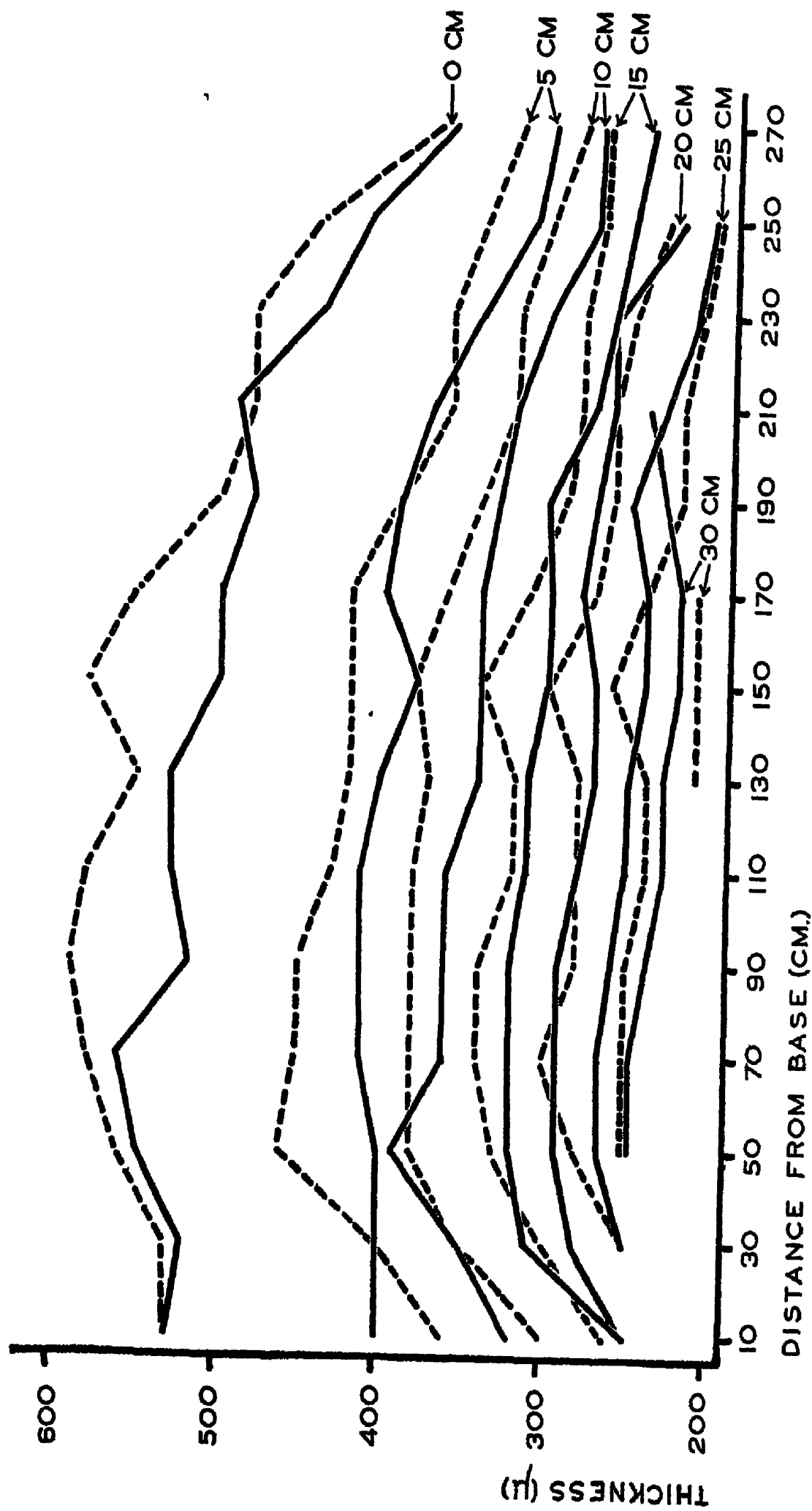


FIG. 1. The pattern of thickness of a leaf of *Musa Balbisiana*. Each line represents thickness at a constant distance from the midrib; solid and dotted lines respectively the broader (right) and narrower (left) lamina-halves.

thickness is attained near the midrib about half the length of the leaf from the base. Across the lamina-half, thickness decreases rapidly at first, then less rapidly until, near the margin, it shows little change. Minimal thickness is found at the margin towards the apex of the leaf. There appears to be a tendency for the broader lamina-half to be thinner, corresponding points on the two halves being compared, but the difference is not entirely consistent and is much less marked or even reversed near the margin. Nevertheless, patterns of thickness over the two halves were similar, but, in view of a possible difference in thickness between them, the broader was invariably sampled in the survey of leaf thickness in relation to ploidy described below. Many other data from series A were plotted and all showed the same pattern as that just described. The results accord well with those of Skutch (1927) and may now be stated to hold generally for *Musa*; and it may be added that the Strelitziaceous species *Heliconia Bihai* (native to Trinidad) and *Ravenala Madagascariensis* both showed similar patterns.

2. A survey of leaf thickness in *Musa*

For comparative purposes it is necessary to select some relatively simple characteristics of leaf thickness in *Musa*. Two obvious choices were the maximal and minimal thicknesses and a third was the mean of the measurements made across the lamina-half where the various curves reached their respective maxima (Fig. 1). These measures were obtained for the samples of series A and the results are summarized in Table I and Fig. 2.

*Ploidy.* Analysis of variance shows that the four categories of ploidy

TABLE I  
*Summary of Results on Leaf Thickness in Relation to Ploidy. Means ( $\mu$ ), Standard Errors, and Percentage Standard Errors for each Class of Ploidy; and Analyses of Variance*

Thickness.	Ploidy.				Analyses of variance.			
	Diploid.	Triploid.	Tetra-ploid.	Penta-ploid.	V.R.	N <sub>1</sub> .	N <sub>2</sub> .	P.
Maximum	489.4 ±21.5 4.4%	596.5 ±21.5 3.6%	628.6 ±23.7 3.8%	510.0 ±44.3 8.7%	7.74	3	48	<0.001
Mean	321.4 ±10.4 3.2%	369.9 ±10.4 2.8%	383.4 ±11.5 3.0%	345.5 ±21.5 6.2%	6.27	3	48	0.01-0.001
Minimum	201.2 ±5.4 2.7%	212.4 ±5.4 2.5%	226.4 ±5.9 2.6%	232.5 ±11.1 4.8%	4.34	3	48	0.01

differ from each other in all three chosen measures of thickness. Essentially, minimal thickness shows a slight but steady increase with ploidy, while mean and maximal thickness increase sharply from diploid to triploid, less markedly from triploid to tetraploid, and show a decline from

tetraploid to pentaploid. Evidently minimal, mean, and maximal thickness show a graded series of reactions to polyploidy: the greater the initial rise, the greater any final depression associated with high polyploidy.

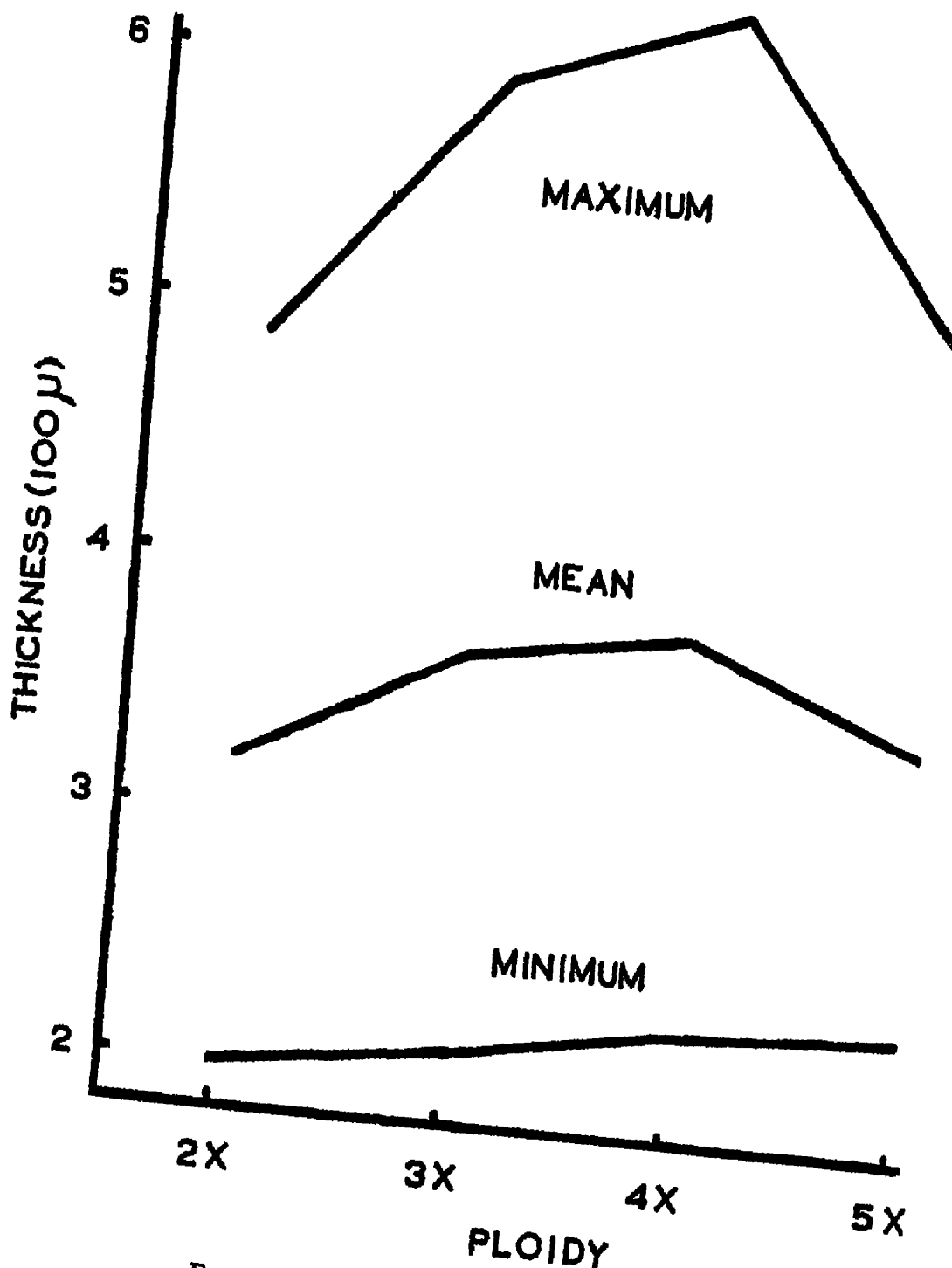


FIG. 2. The relation between thickness and ploidy.

A measure of environmental and genetic variation to which each mean is subject is afforded by the percentage standard errors given in Table I. In every case maximal is more variable than mean, and mean than minimal thickness. Again, therefore, the three characteristics form a graded sequence of what may be called 'susceptibility' to factors influencing thickness.

*The relation between area and thickness.* An examination of Fig. 3 suggests the existence of a positive correlation between area and thickness. Considering first the diploids only, the following results were obtained by fitting straight regression lines of the form  $T = k + bA$ , where  $T$  is thickness in  $\mu$ ,  $k$  a constant,  $b$  the regression coefficient, and  $A$  the area of the lamina-half in sq. cm.:

	$b$	S.E. $b$ .
Maximum . . .	+0.03828	0.002934
Mean . . .	+0.01629	0.001663
Minimum . . .	+0.003107	0.001063

All are significant at the 0.01 level, and so thickness increases with area. Moreover, each regression differs from the other two for

$$t \text{ (maximum-mean)} = 2.066, P = 0.05-0.02$$

$$t \text{ (mean-minimum)} = 2.098, P = 0.05-0.02$$

Again, therefore, maximal, mean, and minimal thickness form a graded sequence, showing progressively slighter susceptibility to the factor influencing leaf thickness. It seems a reasonable inference that this factor is concerned with the different mechanical requirements of leaves of different size, though the mere establishment of a relation between thickness and area is not in itself proof of this.

Similar area-thickness regressions calculated for triploids and tetraploids were as follows:

	Triploid.		Tetraploid.	
	$b$ .	S.E. $b$ .	$b$ .	S.E. $b$ .
Maximum . . .	+0.02022	0.05960	+0.02783	0.01182
Mean . . .	+0.003059	0.02922	+0.008572	0.006158
Minimum . . .	-0.002034	0.01198	+0.001875	0.004330

Of these, the tetraploid maximum is alone significant ( $t = 2.354$ ,  $P = 0.05-0.02$ ). However, with one exception, the regressions are all positive and the same decline in magnitude from maximum to minimum as was found in the diploids is apparent here. Evidently there is an interaction between ploidy and the normal thickness-area relationship such that the latter is clear enough in diploids but decreases as ploidy increases.

### 3. *An empirical equation for leaf area*

From the data of series A a relation between the area of the lamina-half and the product of its length and breadth may be derived. Plotting of  $A$  (area) against  $L$  (length)  $\times$   $B$  (breadth) gave a good straight line (as judged by eye).

$$r = 0.9896 \text{ (} P \text{ very small)}$$

$$\text{and } A = (0.7934 LB - 65.0686) \text{ sq. cm. (S.E.} b = 0.00928).$$

This was calculated for single lamina-halves, but can be applied to whole leaves simply by substituting  $-130.1371$  for  $-65.0686$  as constant.

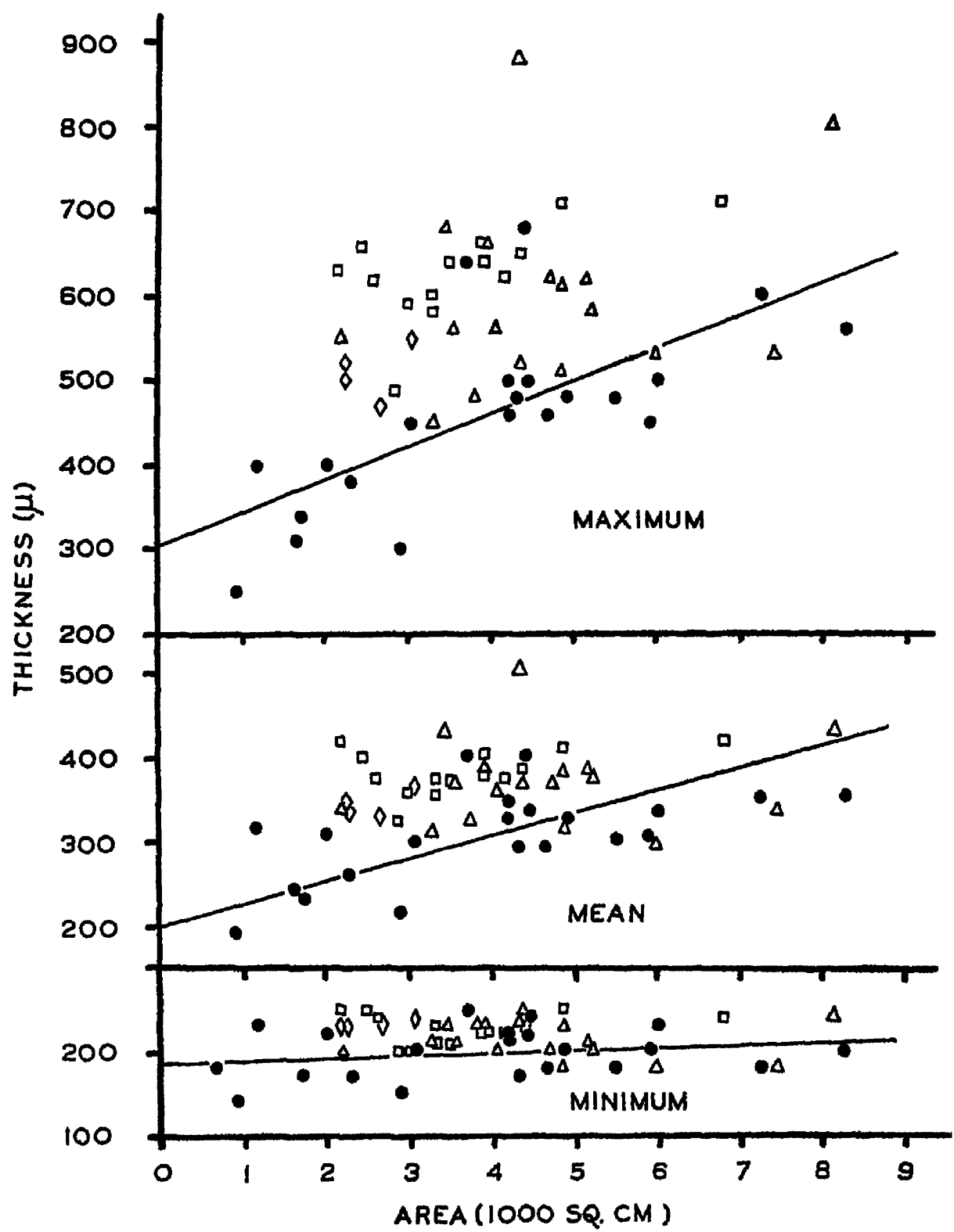


FIG. 3. The relation between thickness and area. Dots = diploids; triangles = triploids, squares = tetraploids; diamonds = pentaploids. Solid lines = area-thickness regression lines for diploids.

4. *The breadth of the lamina-halves in relation to ploidy*

The broader lamina-half of each leaf in series A was scored as ‘left’ or ‘right’, with the following results:

		Broader lamina-half.	
		Left.	Right.
Diploid	.	6	15
Triploid	.	14	3
Tetraploid	.	13	1
Pentaploid	.	4	0

$\chi^2_c$  for the  $2 \times 2$  table formed by combining the polyploid classes is 18.52, which is highly significant; diploids tend to have a right lamina-half broader than the left, while the reverse holds for polyploids.

Two measures of relative breadth were derived from the data of series B, namely: (1) the difference between the breadths of the right and left lamina-halves expressed as a percentage of the total breadth of the lamina and, (2) the ratio of the breadths of right to left lamina-halves, expressed as a percentage (Table II). There are highly significant differences between the

TABLE II  
*The Relative Breadths of the Lamina-halves; Means and Standard Errors*

		Ploidy.†				Analyses of variance.			
Measure.*		Diploid (15).	Triploid (15).	Tetra- ploid (15).	Penta- ploid (6).	V.R.	N <sub>1</sub> .	N <sub>2</sub> .	P.
(1)	$100 \frac{(B_r - B_l)}{(B_r + B_l)}$	+1.00 ±1.117	-2.87 ±1.117	-5.13 ±1.117	-25.50 ±1.766	55.79	3	47	Very small
(2)	$100 \frac{(B_r)}{(B_l)}$	102.0 ±1.97	94.6 ±1.97	91.0 ±1.97	58.3 ±3.16	48.30	3	47	Very small

\* See text:  $B_r$  = breadth of right lamina-half;  $B_l$  = breadth of left lamina-half.  
† In parenthesis, numbers of plants in each category of ploidy.

four categories of ploidy; and both measures of relative breadth give essentially identical results. Triploids have relatively narrower left lamina-halves than diploids; there is no evidence that tetraploids differ from triploids, but pentaploids show a very great and highly significant difference from tetraploids.

The inequality of the two lamina-halves in the leaves of pentaploids was invariably associated with some wrinkling and distortion near the margin of the right (narrow) lamina-half. The same was noticed in a number of tetraploids and a few triploids, although it was never as well marked as in the pentaploids. It was not observed in diploids, the majority of which had a right lamina-half broader than the left. In addition, the narrow and distorted right lamina-half of some polyploids tends to split and wither rather more rapidly than the left.

By contrast, examination of numerous seedlings of various parentage, both diploid and polyploid, showed that the right half was consistently broader

than the left. The effect of polyploidy on relative breadth is, therefore, only manifested in the course of development.

Skutch (1930) states that the veneration of the leaf of *Musa* is constant, the right half being covered by the left. Observation of the collection of *Musa* material at this Institution fully confirms this statement,<sup>1</sup> and herein would seem to lie the explanation of the effect of polyploidy on the relative breadths of the lamina-halves. It seems that the conditions of development of the two halves are different; in the diploid neither side is apparently restricted in growth and the right half is commonly somewhat broader than the left in the mature leaf; in triploids and successively higher polyploids there is a change in proportion causing, after a certain stage in development, successively greater restriction of the growth of the right lamina-half and, thereby, malformation. Probably it is connected with increased thickness of the lamina.

The malformation is distinct from that described by Skutch (1930a) as consequent on impedance to the emergence and unrolling of the leaf; there was no sign of hypertrophy of the hypodermis above the veins, but only a local corrugation of the lamina and sometimes a waviness of the margin that suggested a lack of co-ordination between the growth of the lamina and the development of the marginal vein. Occasionally the faint longitudinal furrows seen on many banana leaves were very strongly marked and even formed lines of weakness of the mature right lamina-half; such furrows result from the folding and compression of the developing leaf (Skutch, 1927).

##### 5. Posture of the leaf and weight of the petiole

The leaves of any banana are practically vertical when first unfurled but they soon take up a declining or drooping posture that increases in degree through the life of the leaf, until it withers and drops off. Thus at any one time the pseudostem bears a sequence of leaves in postures varying according to their ages. However, age is not the only factor affecting the posture of the leaf, for general field observation shows that polyploids tend to have more flaccid leaves than diploids and that the effect increases the higher the ploidy. Furthermore, the flaccidity of the leaves of polyploids is associated with a tendency to breakage of the petiole near the base of the lamina. This association suggests the occurrence of a deficiency of mechanical strength, and the site of breakage may be taken to indicate the region of greatest strain. The samples of series B were designed to throw light on these behaviours.

In order to minimize the effect of age, the central leaf of the sequence was sampled, droop being measured as the ratio of the difference in height between the base and apex of the lamina and the length of the lamina. The former

<sup>1</sup> Twenty emerging leaves of *Maranta arundinacea* L. (Marantaceae) resembled those of *Musa* in this respect. By contrast, material of *Heliconia Bihai* L., *H. psittacorum* L.f. (Strelitziaceae), *Hedychium flavum* Roxb., and *Renealmia bracteosa* Griseb. (Zingiberaceae) was quite inconstant.

quantity may be positive or negative according to whether the apex stands above or below the base, so that the ratio may vary from +1 (leaf vertical) to -1 (leaf hanging vertically downwards). The range observed in this study was +0.89 to -0.56.

Results on the posture of the leaf are summarized in Table III. The regression component (leaf angle on ploidy) accounts for the greater part of

TABLE III

*Posture of the Leaf in Diploids and Polyploids; Means and Standard Errors*  
Ploidy.

Diploid (15).*	Triploid (15).	Tetraploid (15).	Pentaploid (6).
+0.544	+0.173	-0.043	-0.244
±0.0587	±0.0587	±0.0587	±0.0931

\* In parenthesis, numbers of plants in each category of ploidy.

the variance due to ploidy; but there is still a significant residue representing deviations from the regression line:

Item.	Degrees of freedom.	Sum of squares.	Mean square.	Variance Ratio.	P.
Regression .	1	2.2258	2.2258	42.8	0.001
Deviations .	2	1.5684	0.7842	15.1	0.001
Ploidy .	3	3.7942	1.2674	24.4	—
Error. .	47	2.4431	0.0520	—	—
Total. .	50	6.2373	—	—	—

The general impression, therefore, that polyploids have more drooping and flaccid leaves than diploids, and that this effect increases proportionally with ploidy, is fully substantiated.

Fresh weights, dry weights, and percentage dry weights of 30-cm. lengths of petiole are summarized in Table IV and Fig. 4. All analyses of variance

TABLE IV

*Mean Weights of 30-cm. Lengths of Petiole in Diploids and Polyploids*  
Ploidy.

	Diploid* (15).	Triploid (15).	Tetra- ploid (15).	Penta- ploid (6).	Analyses of Variance.			
					V.R.	N <sub>1</sub> .	N <sub>2</sub> .	P.
Fresh weight (gr.) .	50.100	70.167	77.533	40.583	8.63	3	47	<0.001
Dry weight (gr.) .	6.913	8.660	9.580	3.633	4.67	3	47	0.01
Per cent. dry weight	13.846	12.277	12.388	8.762	†			

\* In parenthesis, numbers of plants in each category of ploidy.

† See text.

show significant differences between classes of ploidy; fresh and dry weights reach a maximum at triploidy-tetraploidy and drop sharply with pentaploidy, a behaviour very similar to that found for maximal and mean leaf thickness.

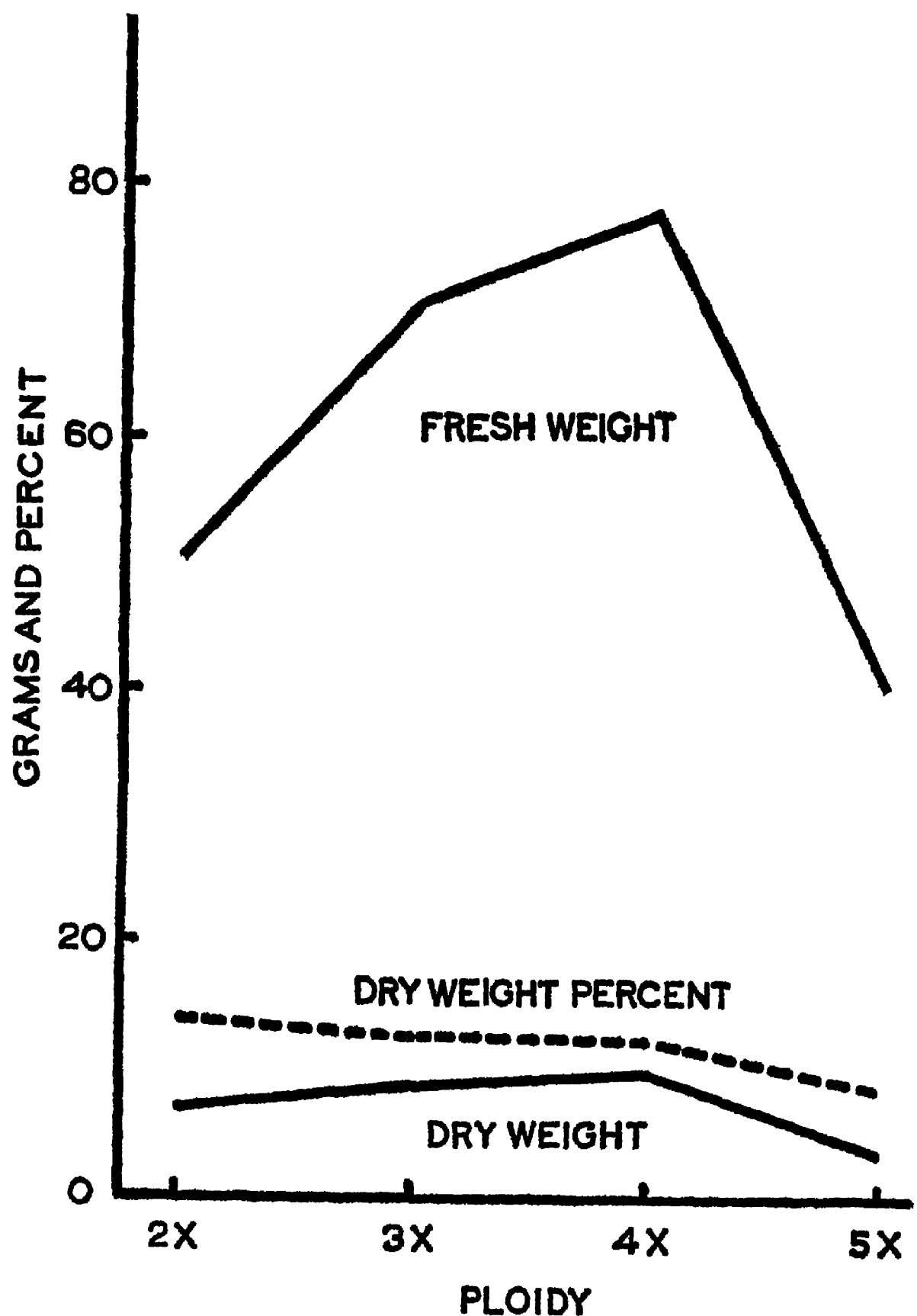


FIG. 4. The relation between weights of 30-cm. lengths of petiole and ploidy.

By contrast, percentage dry weight declines with increasing ploidy, the regression component being highly significant:

Item.	Degrees of freedom.	Sum of squares.	Mean square.	Variance ratio.	P.
Regression .	1	82.6074	82.6074	31.90	0.001
Deviations .	2	28.3175	14.1588	5.47	0.01
Ploidy .	3	110.9249	36.9750	14.28	—
Error .	47	121.7214	2.5898	—	—
Total .	50	332.6463	—	—	—

The percentage dry weight of the petiole may be taken as a measure of its content of material having, at least potentially, a mechanical function. It is therefore established that as this mechanical content declines with increasing ploidy, so also declines the rigidity of the leaf, and it seems a reasonable inference that the one is directly consequent upon the other. Finally, to establish the point, the overall correlation ( $r$ ) between leaf angle and percentage dry weight of the petiole was

$$r = 0.5363,$$

which is significant at the 0.001 level.

#### 4. LAMINAR THICKNESS AND THE ONTOGENY OF THE LEAF

Skutch (1930) showed that differentiation of the leaf of *Musa* is basipetal and that the marginal region of the lamina precedes in development that nearer the midrib. Hence, within a single leaf or lamina-half, thickness and age are inversely proportional, and the three characteristics of thickness chosen in this study represent a developmental sequence—maximal, mean, and minimal thickness, in order of increasing age. It has been shown above that this is also a sequence showing decreasing susceptibility to factors affecting leaf thickness. Thus, the longer the developmental history of a portion of the lamina, the greater the variability of the final product of development, and vice versa.

The effects of polyploidy therefore become cumulatively greater through the ontogeny of the leaf, and this is paralleled by its effect on the relative breadths of the lamina-halves in the ontogeny of the plant as a whole. Elsewhere it has been shown that a similar developmental effect obtains in the case of vigour in *Musa* (Simmonds, 1948). Thus it seems that the phenotypic effects of ploidy have a developmental aspect quite comparable in nature with that already well established for individual genes or complexes of genes.

#### SUMMARY

1. The lamina of the leaf of *Musa* shows a very constant pattern of thickness, being thickest near the midrib about one-half the length of the leaf from the base, and thinnest at the margin towards the apex. Three measures of thickness, the maximum, mean, and minimum, were used.

2. A variety of factors influencing leaf thickness (e.g. polyploidy, genetic and environmental causes, probable mechanical requirements of leaf size)

all influence maximum more than mean and mean more than minimum thickness.

3. This sequence (maximum—mean—minimum) is shown to represent an ontogenetic sequence and a developmental interpretation of the results is advanced; the later the differentiation of a part the more susceptible it is to variation, of whatever origin.

4. In diploids the whole leaf develops freely. In polyploids the growth of one half is variably restricted and a malformation results which increases with the age of the plant and the degree of polyploidy.

5. Leaves of polyploids are more flaccid than those of diploids, and this is correlated with and believed to result from a corresponding decrease in percentage dry weight of the petiole.

I take great pleasure in thanking Professor K. S. Dodds for his help and criticism of this work. I also wish to thank M. Latchman for technical assistance.

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## Note

**An apparatus for the culture of algae at constant temperature.**—In physiological studies with algae it is frequently necessary to grow a large number of cultures under similar conditions of illumination and at a constant temperature. None of the devices described in the literature for the culture of these organisms are very satisfactory for this purpose. Thus only a limited number of culture vessels can be accommodated in a water-bath round an immersed water-cooled lamp, as in the method used by Bristol Roach (*Ann. Bot.*, 1928, xlii. 317–45). Several workers, e.g. Pearsall and Loose (*Proc. Roy. Soc., B*, 1937, cxxi. 451–501) and Chu (*Journ. Ecol.*, 1942, xxx. 284–325), have relied on a constant-temperature room, but this is a convenience that is rarely available. The apparatus described by De (*Proc. Roy. Soc., B*, 1939, cxxvii. 121–39) does not give adequate control of temperature below about 30° C. since there is no provision for cooling the electric lamps used. The devices mentioned in Pringsheim's book on 'Pure Cultures of Algae' (Cambridge, 1946) are not intended to give constant temperature.

The apparatus to be described is relatively simple and has none of these drawbacks. The essential principle in its design is that the lamps are allowed to heat the water-bath in which the cultures are immersed, its temperature being regulated by the controlled admission of cold tap-water.

The main structure is of wood. The upper portion forms a tank, AAA'A' (Fig. 1), with a glass floor, B, let into the wood. After several attempts to make this joint watertight, 'Bostik' adhesive (B.B. Chemical Co., Leicester) was found to be an effective caulking material. Several coats of white waterproof enamel were given to the wood inside the tank and extended for about 2 cm. over the glass. The lower part, in which the sides AA' are left open, carries a board, C, on which the lamps, D, D, D, D, and E, are mounted. C and the insides of the ends, AA and A'A', are painted white. The tank contains a depth of 8 cm. of water, any surplus being carried away by the overflow, F. The culture flasks, of which 48 of the conical 250-ml. size can be accommodated, are carried on a glass shelf, G, resting on the supports, H, H, H, H.

Continuous illumination is provided by five pearl lamps, D, D, D, D, and E, connected in parallel and controlled by a switch, I. They are arranged at calculated distances from the glass shelf, G, and from each other so as to give as nearly as possible uniform intensity of light over the area occupied by the cultures. Using 75-watt 230-volt lamps at D and a 40-watt 230-volt lamp at E, this was found, by means of an Everett Edgcumbe 'Autophotometer', to be about 150 foot-candles (1,615 metre-candles) varying by not more than  $\pm 5$  per cent. from place to place. The effect of the small unevenness in illumination can easily be eliminated by interchanging the positions of the flasks at intervals. An advantage of the apparatus is that the flasks are illuminated from below so that local high light intensities inside the cultures, such as are noticeably produced by illumination from the side, are reduced to a minimum and shading by the cotton-wool plugs is eliminated. Tests with the photometer have shown that, provided direct sunlight is excluded, the lighting conditions are not much altered if the apparatus is kept in an ordinarily lighted laboratory.



The temperature of the water-bath is controlled by a Foote regulator, J. In this, a toluene-mercury system controls the flow of cold tap-water admitted through K at about 0.3 litre/minute so that it runs to waste through L or into the tank through M according as to whether the bath temperature is below or above the temperature for which it is set. Slight modifications of the design given by Findlay (*Practical Physical Chemistry*, 6th edition, London, 1935, p. 33) are necessary. A glass bulb with a hole blown in it has been inserted in the tube L to break the siphon which otherwise made the outflow so rapid as to carry the mercury with it. The bulb containing the toluene has to be horizontal instead of vertical because of the shallowness of the bath, and a side-arm with a tap has been added to facilitate adjustment of the level of mercury. The water in the bath is circulated by the stirrer, N, driven by a 1/30th horse-power 230-volt motor, O, controlled by a 40-watt carbon-filament lamp, P, and a variable resistance, Q, in series, and the switch, R. Although the flasks are immersed only to a depth of 3 cm. in the water, this arrangement gives satisfactory constancy of temperature within them provided that too great a depth of medium, undesirable in any case if adequate aeration is to be obtained, is avoided. It has been found possible to maintain the temperature to within 0.25° in the region of 20° C.; the upper and lower limits at which a constant temperature can be obtained depend on the temperatures of the tap-water and of the laboratory.

The tank is easily cleaned of any growth that may appear, or this can be prevented by the occasional addition of a little sodium hypochlorite to the water.

My thanks are due to Mr. H. J. Page for constructing this apparatus.

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